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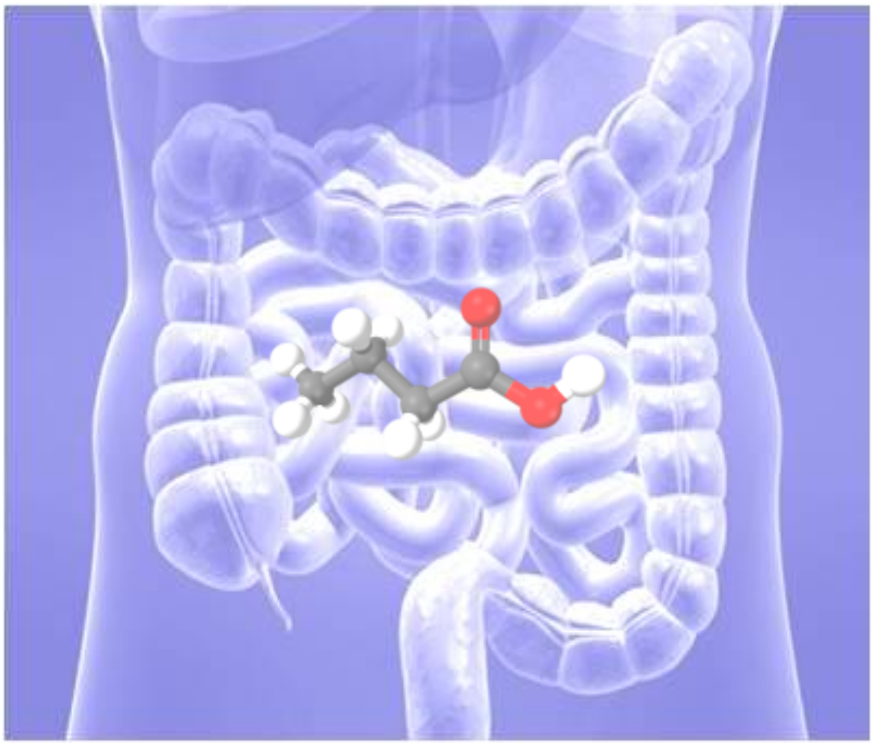
Butyrate transport in the colon epithelium and its repercussion in colorectal cancer



Pedro Filipe Fernandes Gonçalves

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM METABOLISMO - CLÍNICA E EXPERIMENTAÇÃO, APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO.

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ABBREVIATIONS

AA - arachidonic acid

ABC - ATP-binding cassette

ALP - alkaline phosphatase

ASA - acetylsalicylic acid

ATP - adenosine-5'-triphosphate

BCRP - breast cancer resistance protein

BT - butyrate

Caco-2 - human epithelial colon adenocarcinoma cell line

CAF - caffeine

CaM - Ca^{2+}/calmodulin

CAT - catechin

CDCA - chenodeoxycholic acid

CHR - chrysin

CLA - conjugated linoleic acid

COCA - cocaine

COX-2 - cyclooxygenase 2

CRC - colorectal cancer

CTZ - clotrimazole

DCA - deoxycholic acid

DG - 2-deoxy-D-glucose

DHA - docosahexaenoic acid

DNA - deoxyribonucleic acid

EGCG - epigallocatechin-3-gallate

EPA - eicosapentaenoic acid

EPI - epicatechin

ERK 1/2 - extracellular signal regulated kinase 1/2

FA - folic acid

FHC - fetal human colonic epithelial cell line

FTC - fumitremorgin C

GLUT - facilitative glucose transporter

HDAC- histone deacetylase

HDACi -histone deacetylase inhibitor

HIF-1α - hypoxia-inducible factor -1α

IEC-6 - rat non-tumoral intestinal epithelial cell line

LA - linoleic acid

LNA - γ-linolenic acid

MAPK - mitogen-activated protein kinase

MCT1 - H^+-coupled monocarboxylate transporter 1

MDMA – *ecstasy* (3,4-methylenedioxymethamphetamine)

MDR - multidrug resistance

MDR1 - P-glycoprotein

MMR - mismatch repair

MRPs - multidrug resistance proteins

MYR - myricetin

NF-kB - nuclear factor kappa B

NICOT - nicotine

NSAIDs - nonsteroidal anti-inflammatory drugs

OMG - O-methyl-glucose

PKC - protein kinase C

PTK - protein tyrosine kinase

PUFAs - polyunsaturated fatty acids

qRT PCR - quantitative real-time reverse transcription polymerase chain reaction

QUER - quercetin

RESV - resveratrol

ROS - reactive oxygen species

RUT - rutin

SCFA - short-chain fatty acid

SGLT - sodium-dependent glucose cotransporter

SMCT1 - Na^+-coupled monocarboxylate cotransporter 1

TDCA - taurodeoxycholic acid

THC - Δ-9-tetrahydrocannabinol

THEO - theophylline

XN - xanthohumol

INDEX

INTRODUCTION

1. Colorectal cancer: epidemiology, etiology, risk and protective factors and chemoprevention

1.1. Colorectal Cancer

Cancer is the second leading cause of death, after cardiovascular diseases, in occidental countries [1]. Colorectal cancer (CRC) incidence varies considerably across geographical regions; however, it is one of the most common malignancies and cause of cancer death in developed countries [2], such as United States [3] and Europe [4]. In Europe, CRC is the third most common type of cancer in both men and women and the second leading cause of cancer related-death (Figure 1) [2].

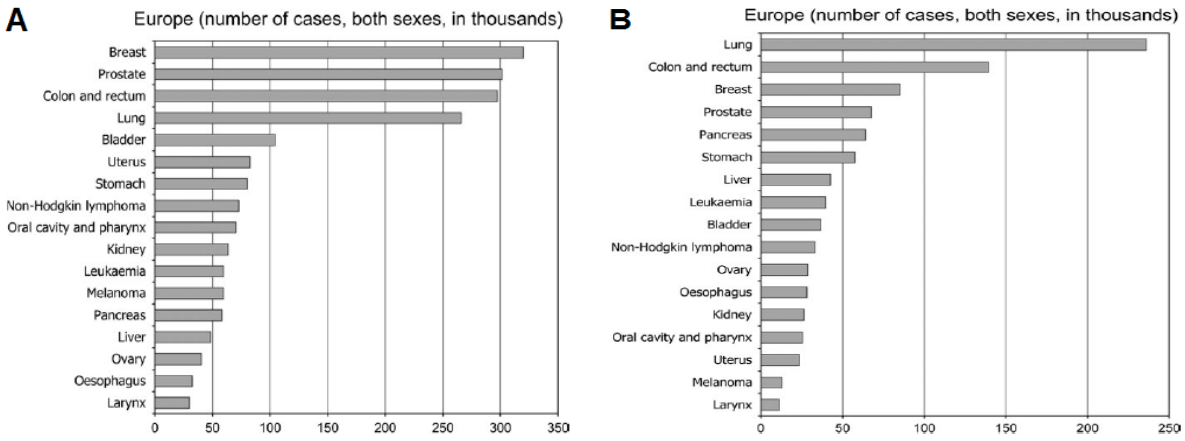

Figure 1. Estimated incidence (A) and mortality (B) of cancer in the European Union (EU25), 2006 (taken from [5]).

The prevalence of CRC has been steadily increasing over the last century (Figure 2), possibly as a result of industrialization and changes in life style/environmental/dietary factors, while mortality rates have declined as a result of efficient screening and surveillance [2].

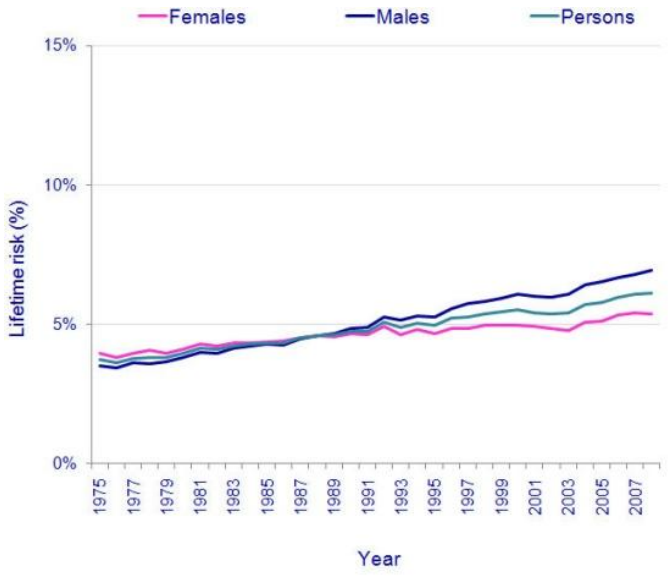

Figure 2. Lifetime risk of colorectal cancer in Great Britain; Cancer Research UK (taken from http://info.cancerresearchuk.org/cancerstats).

CRC includes malignant growths from the mucosa of the colon, rectum and appendix, and most tumors appear in the left side of colon and rectum (Figure 3).

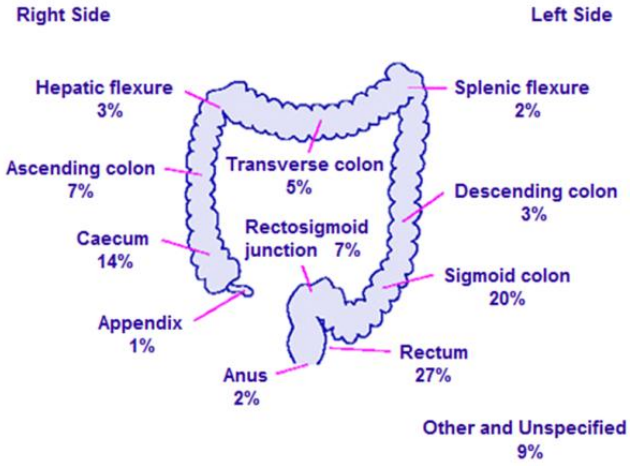

Figure 3. Percentage distribution of cases of colorectal cancer within the large bowel, Great Britain, 2006-2008; Cancer Research UK, (taken from http://info.cancerresearchuk.org).

CRC is traditionally divided into sporadic and hereditary forms [6]. Although there are some well- recognized genetic causes of CRC, the vast majority of cases (≈95%) are considered to be sporadic [7]. Instead, only approximately 5% of cases are associated with one of two genetic syndromes, namely familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome). FAP is associated with an inherited genetic mutation in the tumor suppressor gene adenomatous polyposis coli (APC), and HNPCC to mutations in genes associated with DNA mismatch repair [7].

1.2. Risk and protective factors

Risk factors for CRC include age, family history and environmental and dietary factors [8]. In younger populations, the risk for developing CRC is very low but the probability of developing CRC rises sharply with age (Figure 4) [8]. Also, a family history of CRC (particularly with relatives diagnosed under the age of 45 years) is associated with an increased risk of developing CRC [9]. Evidence from migrating populations [10] suggests a strong effect of environment on CRC risk [11,12]. Chronic inflammatory bowel disease (Crohn's disease and ulcerative colitis) is also associated with an increased risk for CRC [1]. Also, epidemiological studies have highlighted the role of diet and lifestyle in CRC risk: positive correlations have been reported for high caloric intake, high consumption of red meat (especially if burned) and saturated fat, high alcohol intake as well as cigarette smoking, obesity and diabetes [8,13,14], and inverse correlation between CRC and the intake of fish (rich in n-3 polyunsaturated fatty acids), fruits and vegetables [15-18]. Studies estimate that nutritional factors are responsible for 70-90% of CRC cases, and that diet optimization will prevent most of them [19,20]. Other non dietary environmental lifestyle and supplementary factors with protective effects include moderate to vigorous physical activity, hormone replacement therapy in postmenopausal women, and the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin [21,22].

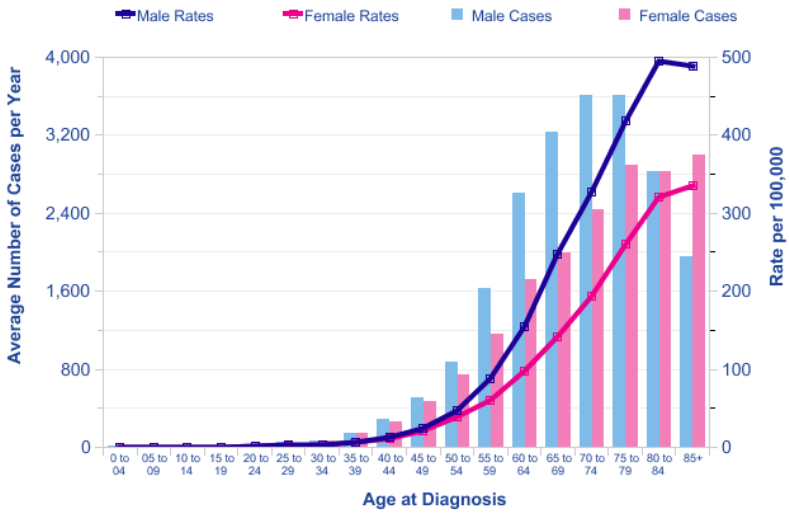

Figure 4. Average number of new cases per year and age-specific incidence rates per 100,000 population; Cancer Research UK (taken from http://info.cancerresearchuk.org).

As stated, diet and other environmental risk factors play important roles in CRC development [8]. However, only a small proportion of individuals exposed to those risk factors will develop CRC, while many individuals without those risk factors develop CRC. This suggests

that genetic factors also play an important role in colorectal carcinogenesis [23]. So, CRC development is determined by a complex interaction between genetic and specific environmental/life style risk factors with different degrees of involvement [23].

1.3. Colorectal carcinogenesis

CRC results from the cumulative effect of multiple sequential genetic and epigenetic alterations in colorectal epithelium (multistep carcinogenesis) (Figure 5), generally acquired (and not inherited) [24,25]. Multiple molecular pathways appear to exist in CRC, but three major independent pathways have been described: the chromosomal instability pathway (CIN) (adenoma-carcinoma sequence) (Figure 5), which is characterized by allelic loss, a pathway involving microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathway [26].

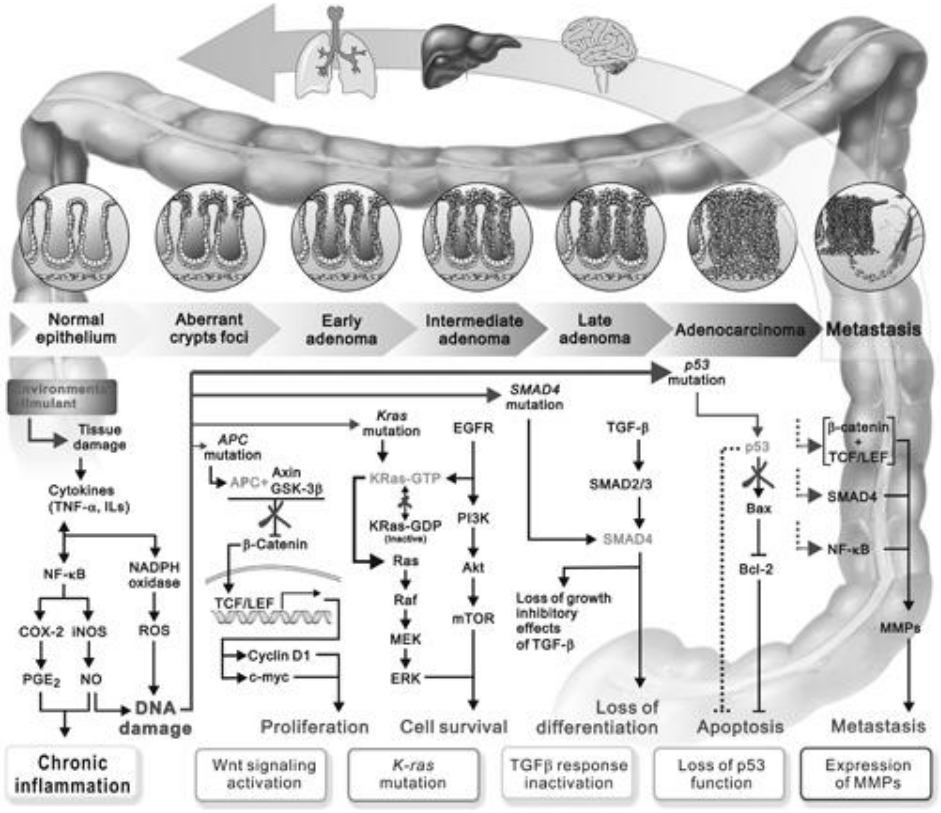

Figure 5. Mechanisms and molecular events that characterize the transition from normal epithelium to CRC (taken from [27]).

The large majority of CRC cases develop from the CIN pathway [26], where colorectal polyps (also referred to as adenomas) are an intermediate step in the development of adenocarcinoma [28,29]. Indirect evidence suggests that adenomas may be present for 10

years or more before malignancy development [30,31]. Adenomas smaller than 3 mm in diameter are classified as having very low malignancy risk and rarely develop to malignancy [28]. However, adenomas are found to coexist with cancer in approximately 30% of cases [32,33]. So, current efforts are directed towards detection and removal of adenomas. About 90% of total adenomas can be observed and removed by colonoscopy. Therefore, colonoscopy has become a part of routine clinical practice and is the method of choice for CRC screening and prevention [34,35].

1.4. Chemoprevention strategy

Although the efficacy of colonoscopy (and fecal occult blood testing) in reducing CRC incidence and mortality is well established, there are some major pitfalls. First, the positive impact of colonoscopy is largely operator-dependent [36-38]. Second, colonoscopy seems to provide more protection against distal rather than proximal colon cancer [38-40]. So, because CRC prevention by colonoscopy has some limitations, chemoprevention might be an alternative choice for reducing the incidence of CRC [41]. Chemoprevention is the use of synthetic or natural compounds, in pharmacological doses, to reduce the risk of development or recurrence of cancer [42]. Many natural dietary compounds present in fruits and vegetables, including fiber, short-chain fatty acids (e.g. butyrate, which is a product of dietary fiber decomposition in large intestine), folate, L-methionine, selenium, vitamin D, calcium and phytochemicals have been isolated and their health promoting properties have been demonstrated [42,43].

2. Butyrate in CRC

Dietary fiber (particularly from cereal and whole grains [44]) is one of the most promising candidates for a protective role in CRC, with strong support from both epidemiological and experimental animal studies [45-48]. Dietary fiber exerts a protective role against CRC through a variety of mechanisms [42,43]. Short-chain fatty acids (SCFAs; acetate, propionate and butyrate) are organic acids produced in the intestinal lumen by bacterial fermentation of mainly undigested dietary fiber [49]. Butyrate (BT) is an important mediator in the observed protective effect of fermentable dietary fiber in the prevention/inhibition of colon carcinogenesis [50-52]. BT is the main energy source for colonocytes [53] and promotes growth and proliferation of normal colonic epithelial cells [54,55]. However, BT inhibits colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis

and inhibiting cell proliferation) [56,57]. Also, several well-designed animal models have demonstrated a protective effect of BT on colorectal carcinogenesis [58-63]. Moreover, there is an inverse relationship between the levels of BT in the human colon and the incidence of CRC [64], as well as an increased incidence of tumors in the distal colon, where the concentration of BT is lower, suggesting an inverse relationship between BT and CRC [65]. A role for BT in the development of CRC has been also supported by the observation of a downregulation of BT transporters (monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1)) in CRC, causing a reduction in BT uptake by colonic epithelial cells [66-68].

2.1. Mechanisms involved in the anticarcinogenic effect of BT

The mechanism by which BT inhibits colon carcinogenesis seems to involve various effects on gene expression, which are mainly attributed to its capacity to act as a histone deacetylase inhibitor (HDACi), leading to hyperacetylation of histones [69,70]. Histone hyperacetylation leads to a more relaxed chromatin structure, and thus facilitates transcription factor access to the promoter regions of certain genes, without directly initiating gene transcription [71,72]. HDAC inhibitors are critical epigenetic regulators, and a new class of anticancer agents by virtue of their ability to arrest cell proliferation and promote cell differentiation or stimulate apoptosis in tumoral cells [72-74]. However, it is likely that BT has other intracellular targets, including alteration of DNA methylation [75], histone methylation [76], hyperacetylation of nonhistone proteins [77], selective inhibition of histone phosphorylation [78], regulation of the expression of micro-RNA (miRNA) [79,80], and modulation of intracellular kinase signaling [81-84].

More recently, it was demonstrated that BT can also elicit biologic effects on colonic epithelial cells without entering the cells [85]. BT is a physiologic agonist of GPR109A, a G-protein-coupled receptor which is abundantly expressed in the apical membrane of mouse and human colonic epithelial cells [85]. GPR109A acts as a tumor suppressor in colon, and its expression is silenced in human CRC, in a mouse model of CRC and in colon cancer cell lines [85]. SCFAs were also reported as ligands for GPR41 and GPR43 [86-88], activating these receptors in a concentration-dependent and specific manner [87,89]. GPR43 has been shown to be present in both human and rat colon [90], and GPR43 expression is markedly reduced and, in some cases, completely lost in colon adenocarcinomas and in established colon cancer cell lines [91]. The expression pattern of GPR43 suggests that the loss of GPR43 expression is related to the transition from a benign to a malignant carcinoma.

BT influences several important processes in tumorigenesis, including cell proliferation, apoptosis, angiogenesis, immunosurveillance, oxidative stress and inflammation. In colon cancer cells, BT elicits an anticarcinogenic effect by induction of apoptosis [56,92], inhibition of proliferation and promotion of a more differentiated phenotype [93,94]. BT is able to block cell proliferation, mainly in the G1 phase of the cell cycle [95], by inducing a decreased expression of the proto-oncogenes c-src and c-myc [96,97] or an increase in $p21^{Waf1/Cip1}$ and cyclin D expression [98,99], via inhibition of HDACs. However, a recent report showed that BT affects $p21^{Waf1/Cip1}$ gene expression via decreased expression of the miR-106b gene family [79]. BT also induces G2/M cell-cycle arrest [100]. Additional effects of BT on cell apoptosis involve the intrinsic or mitochondrial apoptotic pathway [101,102], affecting Bcl2 family proteins, e.g., upregulation of (pro-apoptotic) BAK and downregulation of (anti-apoptotic) BclxL [103,104]. However, BT can also induce extrinsic apoptosis pathway [101].

Angiogenesis, the formation and growth of new blood vessels from preexisting microvasculature, is a key stage in tumor growth, invasion, and metastasis. It has been suggested that BT inhibits tumor-induced angiogenesis through modulation of vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1α and neuropilin-1 (NRP-1) [105,106]. In addition, BT decreases the metastatic and invasive potential of cancer cells by decreasing the activity of pro-metastatic matrix metalloproteinases (MMP-2) and other metastasis-associated genes as well as by inducing metastasis suppressors [107-109].

Oxidative stress has the potential to affect a large array of carcinogenic pathways, because its targets, including DNA, RNA, lipids, and proteins, are involved in enhanced malignant transformation and proliferation of initiated cells [110]. BT inhibits oxidative stress, since incubation of isolated rat [111] or human [112] colonocytes with BT showed a significant reduction in H_2O_2-induced genotoxicity. BT also reduced 4-hydroxynonenal-induced genotoxicity in colon cells [113]. BT does not act as a free-radical scavenger due to its chemical structure. However, BT may act as a secondary antioxidant by affecting the levels of enzymatic or non-enzymatic antioxidant systems and DNA repair systems. Indeed, BT increases antioxidant GSH levels [46,112,114] and enhances glutathione-S-transferase [115] and catalase activities [116] thus reducing oxidative stress levels. Moreover, cells defective in DNA mismatch repair (MMR) genes generate mutations at a rate 1000-fold greater than observed in normal cells, being the hMLH1 (human mutL homolog 1) the most frequently MMR gene silenced in CRC [117], and BT was found to reduce hMLH1 promoter methylation in CRC cells [118], and to increase the accessibility of DNA repair enzymes to chromatin [112].

Inflammatory bowel disease (chronic colitis or persistent inflammation in the colon) is associated with a higher risk of CRC [119]. Inflammation promotes CRC development by

creating an inflammatory microenvironment during tumor tissue formation. One mechanism to explain the contribution of inflammation to carcinogenesis is oxidative stress, and interestingly BT inhibits oxidative stress, as shown above [111,112]. Another mechanism to explain the role of inflammation in cancer is the production of cytokines by inflammatory cells. The inflammatory and immunosuppressive cytokines and chemokines not only promote proliferation, angiogenesis, invasion, and metastasis but also suppress the host's immune system and facilitate tumor growth and CRC development [110]. Interestingly, BT has been shown to reduce inflammation by inhibition of nuclear factor kappa B (NF-kB) activation [120-122] and by upregulation of peroxisome proliferator-activated receptor-ɤ (PPARɤ) [123,124]. Moreover, cyclooxygenase-2 (COX-2) overexpression, which plays an important role in the inflammation-carcinogenesis pathway of CRC, is suppressed by BT [125-127].

3. Absorption of BT

Because many cellular effects of BT are dependent on its intracellular concentration (e.g. inhibition of histone deacetylases), knowledge on the mechanisms involved in its membrane transport seem particularly important. Absolute concentrations of BT in human faeces were found to range from 11 to 25 mM [128,129], and BT is preferentially absorbed in the proximal part of colon where the highest luminal concentration occurs [130-132]. BT is a weak acid (pKa=4.8), more than 90% existing in the ionized form under physiological conditions in the colon (pH 5.5–6.7), and requiring a transporter protein for absorption [133-135]. Several different mechanisms for BT uptake across the apical membrane of colonocytes have been proposed, including simple diffusion of the undissociated form (through the lipid membrane (in the distal colon)) [133-135], counter-transport with bicarbonate (BT/HCO_3^- exchanger) [136,137] and transport by monocarboxylate transporters [138,139]. The two major monocarboxylate transporters identified for BT absorption across the luminal membranes of colonocytes are the H^+-coupled monocarboxylate transporter 1 (MCT1), a member of SLC16 gene family [138-140], and the Na^+-coupled monocarboxylate transporter 1 (SMCT1), a member of the SLC5 gene family [138,139,141]. The molecular identity of the non-electrogenic pH-dependent BT/HCO_3^- exchange is yet unknown [142,143].

3.1. Monocarboxylate transporter 1

The monocarboxylate transporter (MCT) family is composed by 14 members, and belongs to the major facilitator superfamily, being encoded by the SLC16 gene family (reviewed in [144]). Although being a family of 14 members, only the first four (MCT1-MCT4) have been demonstrated experimentally to transport monocarboxylates [145-147]. To function, MCT translocates a proton through the plasma membrane together with a monocarboxylate, by an ordered mechanism in which H^+ binding is followed by monocarboxylate binding to the protonated transporter [148]. The monocarboxylate flux direction depends on the chemical gradients for H^+ and monocarboxylates across the membrane [147].

The MCT1 (SLC16A1) gene was cloned in 1994 [149] and the structural gene organization as well as isolation and characterization of SLC16A1 promoter was then achieved [150]. MCT1 functional protein is composed by 500 amino acids (Figure 6), is quite well conserved (e.g. the protein sequences of rat and humans show 83% identity and 87% similarity) and is ubiquitously expressed in almost all tissues [151]. MCT1 is composed of 12 transmembrane alpha helices (TMs) with a large loop between TMs 6 and 7 and the C and N termini facing the cytosol [152,153]. The N-terminus is more conserved among different MCTs isoforms and is believed to be more important for energy coupling, membrane insertion and/or correct structure maintenance, whereas the C-terminal domain may be more important for determination of substrate specificity and binding [154].

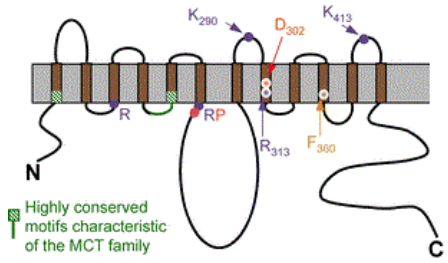

Figure 6. MCT1 protein diagram (taken from [144]).

MCT1 transports a variety of substrates including short-chain (C2-C5) unbranched aliphatic monocarboxylates (e.g. BT), and monocarboxylates with C2 or C3 substitutions (excluding amino/amido substitutions) (e.g. pyruvate, L-lactate, acetoacetate and β-hydroxybutyrate) [155,156]. More hydrophobic ketoacids derived by transamination of amino acids may also be transported by MCT1: this includes phenylpyruvate (from phenylalanine), α-ketoisocaproate (from leucine), α-ketoisovalerate (from valine), and α-keto-β-methylvalerate (from isoleucine) [144]. However, numerous drugs containing a carboxyl group in their

chemical structure and/or weak organic acids compounds may be potential substrates for MCT1.

3.1.1. MCT1 regulation

Several aspects of MCT1 regulation are poorly understood. However, it is known that MCT1 is regulated at various points including transcriptional and post-transcriptional regulation, that affect protein amounts, and regulation of transporter activity (e.g. by chaperone proteins).

Studies indicate that MCT1 is regulated at various points during gene expression. The SLC16A1 promoter has putative binding site sequences for the transcription factors upstream stimulatory factor (USF) 1 and 2, NF-κB, activated protein 1 and 2 (AP1 and AP2) and stimulating protein-1 (Sp1) [150]. Indeed, USF1 and USF2 have been described as potential repressor proteins for MCT1 [157], and NF-κB pathway is involved in BT-induced MCT1 upregulation [158], while AP2 has been associated to protein kinase C (PKC)-dependent stimulation of the SLC16A1 promoter [159]. Also, the co-activators peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) [160] and peroxisome proliferator-activated receptor alpha (PPARα) [161,162] have been associated with MCT1 upregulation. MCT1 is also under post-transcriptional regulation by microRNAs. The MCT1 untranslated region is a target for three microRNAs (miR-29a, miR-29b, and miR-124), and recently, miR-29a and miR-29b have been described to silence MCT1 expression [163].

At the protein level, MCT1 expression varies along the human digestive tract: very low MCT1 expression is found in the small intestine but its expression increases in the colon with maximal levels in the distal segment, being confined to the upper regions of colonic crypts and to apical side of epithelial cells [164,165]. There is a profound diurnal rhythmicity in the expression of MCT1, with a maximal expression occurring before feeding phase [166]. Moreover, an increase in MCT1 protein levels was found in the gastrointestinal tract after feeding with fiber [167], and BT also induces MCT1 protein expression [158,168-170].

Accessory proteins also regulate MCT1 protein function. MCT1 is associated with CD147 (also known as basigin, EMMPRIN, OX-47 or HT7), a broadly distributed plasma membrane glycoprotein [171]. This ancillary protein is involved in the regulation of MCT1 activity and localization. For example, in MCT1-transfected cells, co-transfection with CD147 increased the expression of MCT1 in the plasma membrane [172,173]. On the other hand, MCT1 was also shown to be a regulator of CD147 maturation and trafficking to the plasma

membrane [174,175]. So, these two proteins must remain associated for MCT1 transport activity to be maintained (Figure 7) [172,176].

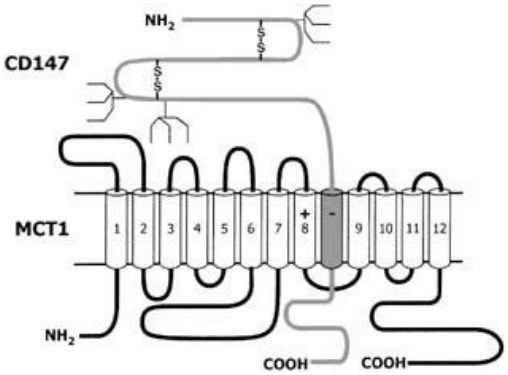

Figure 7. Monocarboxylate transporter 1 (MCT1) and CD147 predicted interaction. The interaction between CD147 and MCT1 is proposed to involve an L-arginine residue within transmembrane segment 8 of MCT1 and an L-glutamic acid residue in the transmembrane segment of CD147 (taken from [177]).

The transmembrane H^+ concentration gradient, or transmembrane pH differential, is a major factor that controls MCT1 function [156,178]. MCT1 is thus functionally coupled to proteins involved in acid/base regulation. Carbonic anhydrase (CA) plays a supportive role in pH regulation, as it clearly contributes to increased extracellular H^+ concentration, thereby enhancing MCT1 transport activity [179]. Morever, carbonic anhydrase II, by direct binding to the C-terminal of MCT1 (possibly inducing an allosteric conformation change) also increases MCT1 transport activity [179]. Finally, Na^+/HCO_3^- cotransporter (NBC) and Na^+/H^+ exchangers (NHE) also enhance MCT1 transport [180].

Several chemical agents are known to inhibit MCT1 activity. Classical reversible inhibitors of MCT1 fall into four broad categories [144]: (1) Bulky or aromatic monocarboxylates like phenyl-pyruvate and α-cyano-4-hydroxycinnamate (CHC); (2) inhibitors of anion transport such as 5-nitro-2-(3-phenylpropylamino)benzoate and niflumate; (3) thiol (e.g. *p*-chloromercuribenzene sulphonate (pCMBS)), and amino (e.g. pyridoxal phosphate and phenylglyoxal) reagents; (4) amphiphilic compounds with widely divergent structures, like phytochemicals [181-183]. However, none of these MCT classical inhibitors is either MCT-specific or MCT isoform-specific. AstraZeneca® newly developed immunomodulatory compounds appear to be MCT1 isoform-specific [184].

3.1.2. MCT1 in CRC pathophysiology

The first report on MCT1 protein expression in human tumor samples described a decrease in MCT1 expression in colonic transition from normality to malignancy [66,185]. Later, evidence for MCT1 downregulation was also observed in other types of cancers

[66,186]. The decrease in MCT1 expression results in reduced uptake, metabolism and effects of BT in the colonocytes. The loss or silencing of MCT1 has been demonstrated to correlate with (a) transition from normality to malignancy in colonic epithelium [66], (b) deregulation of BT-responsive genes involved in differentiation and apoptosis [185,187] and (c) important metabolic switch from BT β-oxidation to glycolysis [188], with a simultaneous increase in GLUT1 expression [66,189] and production of high levels of glycolytic metabolites [190]. Furthermore, MCT1 protein is also downregulated in inflamed mucosa of inflammatory bowel disease patients, in animal models of inflammation, and in response to the proinflammatory cytokines IFN-ɤ and TNF-α [191]. Interestingly, treatment with infliximab (an anti-TNF-α monodonal antibody) significantly increased MCT1 mRNA levels in inflamed colon of Crohn's disease patients [192]. The reduction in uptake of BT which occurs during inflammation may contribute to the fact that inflammatory bowel disease is associated with an elevated risk of CRC [191,193].

3.2. Sodium-coupled monocarboxylate transporter 1

SLC5A8 transporter is a member of the sodium solute symporter family (SLC5). It was first cloned and recognized as a passive transporter by Rodriguez et al. (2002) in the search for the sodium iodide symporter [194]. This transporter is encoded by the SLC5A8 gene (Figure 8) localized at chromosome 12q23.1 [67]. The SLC5A8 gene promoter has been also characterized [195]. SLC5A8 encodes a protein with 610 amino acids [196] expressed abundantly in the apical membrane of the ileum and colon [68,165,197,198]. In both humans and mice, SLC5A8 protein levels are highest in distal colon, followed by proximal colon and ileum [193].

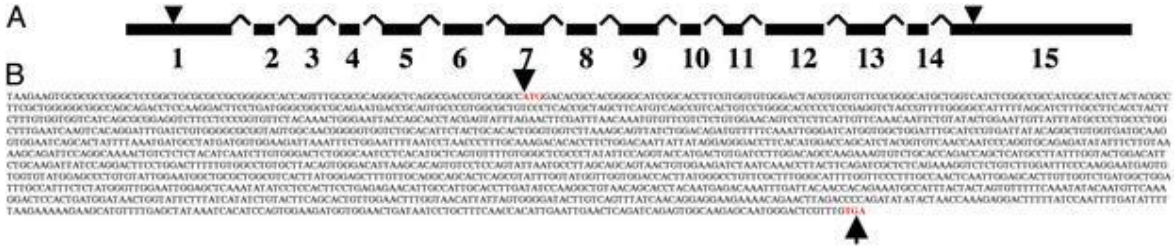

Figure 8. SLC5A8 gene. (A) The genomic structure of the SLC5A8 gene. Black boxes represent exons, and arrows represent the start and stop codons. (B) The nucleotide sequence of the SLC5A8 coding region (taken from [67]).

Recently, SLC5A8 has been characterized as a Na^+-coupled BT transporter [199,200] being thus also referred as sodium-coupled monocarboxylate transporter 1 (SMCT1) [201]. BT is cotransported with Na^+ into the cell, following the inward electrochemical gradient for Na^+ ions maintained by the Na^+/K^+-ATPase [199,200]. The transport is electrogenic, due to $3Na^+$:1BT stoichiometry that results in the transfer of net positive charge into cells [202]. The

substrate specificity of SMCT1 and MCT1 is very similar [144]. SMCT1 transports a variety of monocarboxylates such as lactate, pyruvate and short-chain fatty acids [198,199], ketone bodies [203], nicotinate structural analogs [204,205], pyroglutamate (amino acid derivative) [206] and benzoate and its derivatives (salicylate and 5-aminosalicylate) [205].

3.2.1. SMCT1 regulation

Information on the regulation of SMCT1 expression and function in the intestine is very limited. It is known that ob/ob mice (an animal model of obesity) show a decrease in SMCT1 protein levels [207], and SMCT1 is also downregulated by inflammation [193]. Studies have shown that SMCT1 expression is frequently silenced in aberrant crypt foci (the earliest detectable morphologic abnormality of the colonic epithelium), colon adenomas, colon cancers and colon cancer cell lines, suggesting that SMCT1 silencing is an early event in colon tumorigenesis [67,201]. SMCT1 is also silenced in cancers of the thyroid, head and neck, breast, stomach, prostate, pancreas and blood [67,208-214]. Interestingly, CRC patients often have chromosome 12q allelic loss, which contains the SLC5A8 gene [215,216]. So, SMCT1 was proposed to function as a tumor suppressor, the ability of this transporter to mediate the entry of BT into colonocytes underlying its potential tumor suppressor function [67,141,201]. Of note, combination of upregulation of matrix metalloproteinases 7 and SMCT1 downregulation is an optimal biomarker for identifying CRC cases [217]. Also, SMTC1 activity was positively correlated with CRC remission and patient survival [68].

SMCT1 expression is decreased in the absence of gut commensal bacteria [218] and by TNF-α [193] and increased by the probiotic *Lactobacillus plantarum* [193] and by activin A (a member of the transforming growth factor-β (TGF-β) superfamily) [219]. Finally, SMCT1 activity is known to be inhibited by some non-steroidal antiinflammmatory drugs (NSAIDs) (ibuprofen, ketoprofen, fenoprofen and naproxen, with ibuprofen being the most specific and potent inhibitor of SMCT1) [205], and stimulated by some other NSAIDs (diclofenac, meclofenamate and sulindac) [220].

4. From tumor cell metabolism to CRC therapy

BT is considered to be the main energy source for colonocytes, accounting to about 70% of total energy utilization (Figure 9) [221].

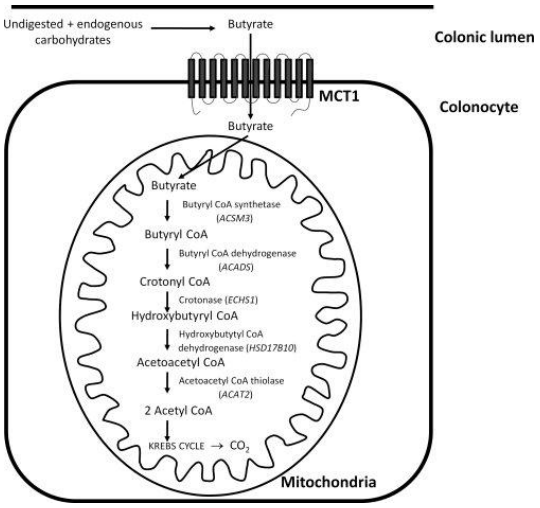

Figure 9. Overview of BT metabolism (uptake and oxidation) in colonocytes. In this figure, taken from [222], BT uptake is represented as MCT1-mediated only, because SMCT1-mediated BT uptake in the native human intestine or in intestinal epithelial cell lines had not yet been shown.

However, CRC often exhibits an altered cell metabolism when compared to normal colorectal tissues. More specifically, CRC shows an increase in the rate of glucose uptake, via an upregulation of the facilitative glucose transporters (e.g. GLUT1) [223], an increase in the expression levels and activity of glycolytic enzymes and glycolysis (2- to 17- times) (Figure 10) [221,224,225]. As a result, CRC has a markedly enhanced rate of anaerobic glycolysis (this is referred as the Warburg effect - i.e. the continued use of the glycolytic metabolic pathway even in the presence of adequate oxygen to support mitochondrial oxidative phosphorylation), and rapidly convert the majority of glucose into lactate [221,226]. CRC cells also show a reduction in BT uptake, which is consistent with the reduction in MCT1 and SMCT1 expression [221,226]. This agrees with the observation that increased intracellular glucose concentration (due to an increase in glucose uptake) downregulates MCT1 expression in CRC cells [227]. This may explain why CRC cells are less dependent on BT as energy source and, therefore, more reliant on glucose.

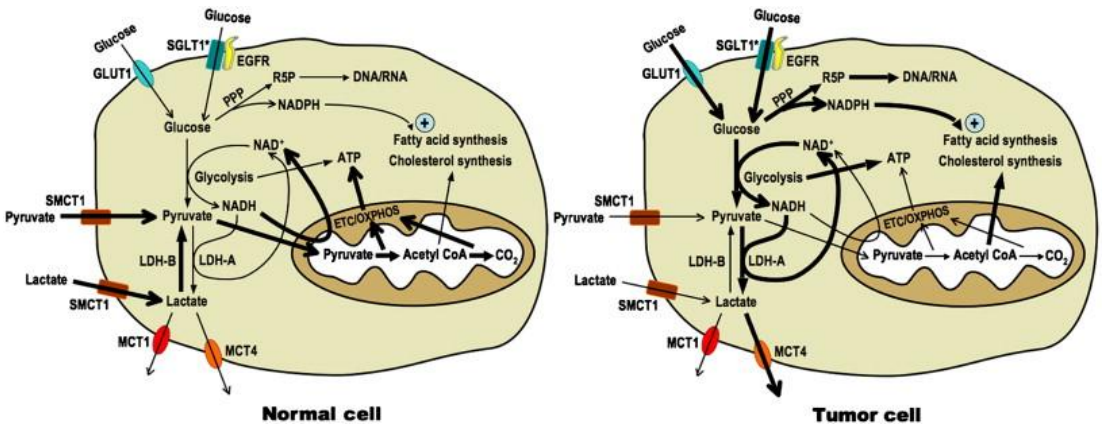

Figure 10. Transport and metabolism of glucose in normal cells versus tumor cells (taken from [228]).

The high rate of glucose uptake associated with increased glycolytic fluxes has been exploited clinically for the detection of malignant tumors [229,230]. Indeed, as tumor cells are metabolically more active than untransformed cells, ^{18}F-fluorodeoxyglucose (FDG) is preferentially taken up by tumor cells resulting in a hot spot on positron emission tomography (PET) imaging, thus providing a powerful diagnostic tool with a high sensitivity and specificity [231].

Additionally, the increased glycolytic metabolism in CRC provides multiple potential targets for treatment strategies [232]. Briefly, the potential therapeutic strategies may target glycolysis inhibition, that will result in cancer cell death, or promote oxidative phosphorylation [232]. In relation to the first possibility, glycolysis can be dampened by reducing glucose availability through inhibition of its uptake or by selective inhibition of glycolytic enzymes. Although the main controlling steps of glycolysis are glucose transporters (GLUTs) and hexokinases (HK) [233], experimental approaches usually yielded more than a single proposed rate-limiting step for inhibition of glycolysis (Figure 11) [233]. Indeed, several distinct components of the glycolytic pathway have been targeted for therapy development (Figure 11) although relatively few have been carried through to *in vivo* experiments and even fewer to clinical trials [234]. Various glycolytic inhibitors with potential anticancer activity have been identified. Lonidamine, a derivative of indazole-3-carboxylic acid, is a specific inhibitor of hexokinase II and was one of the first glycolysis inhibitors to be tested in cancer therapy [235]. The glucose analog 2-deoxy-D-glucose (2-DG) has been most widely investigated for glucose metabolism targeting. 2-DG competitively inhibits glucose transport and phosphorylation by hexokinases [236-238]. 3-Bromopyruvate, an alkylating agent, has been demonstrated to be a powerful and specific antitumoral agent [239-241]. 3-Bromopyruvate inhibits cellular energetic metabolism, both at glycolysis and mitochondria levels, depleting the whole ATP cell factory [242]. Clotrimazole, an antifungal imidazole derivative, can induce detachment of glycolytic enzymes from mitochondria and thus inhibits glycolysis [243].

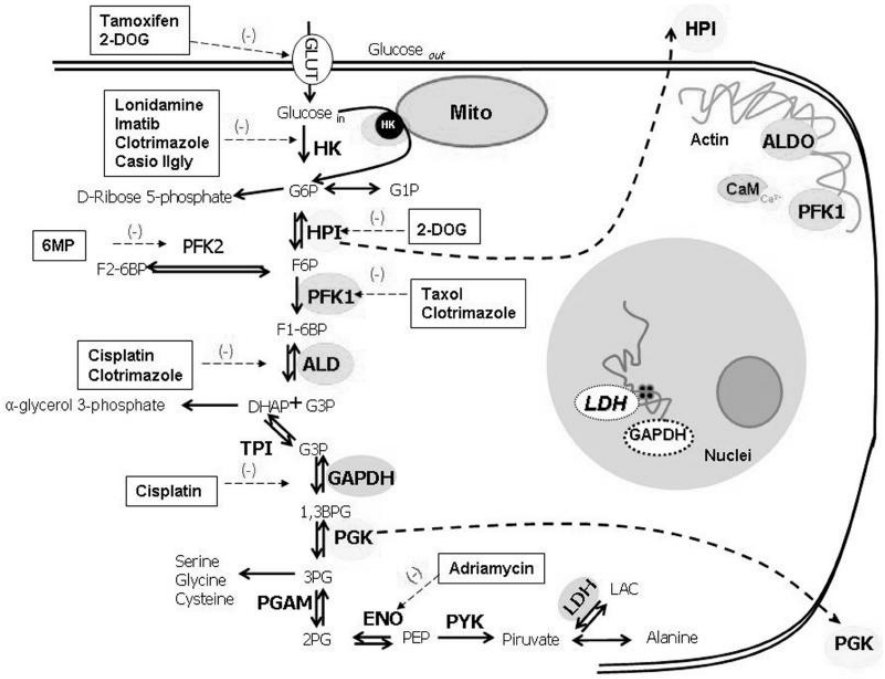

Figure 11. Glycolytic enzyme targeting by anticancer drugs (taken from [221]).

Tumoral cells convert the majority of glucose into lactate, and to maintain a permissive intracellular pH, high glycolytic rates and ATP levels, cells must efficiently export lactate [244]. MCTs contribute to intracellular pH regulation by co-transporting lactate together with a proton [147]. MCT4 is a low-affinity/high capacity lactate transporter (K_m of 20–30 mM) associated with the export of lactate in cells with high glycolytic rates [147] and known to be upregulated in cancers and in tumor cell lines [245,246]. Interestingly, oxygenated tumor cells (located at the vicinity of blood vessels) prefer using lactate over glucose as the primary energy source for oxidative metabolism [247-250]. Recently, a symbiosis between anaerobic glycolytic cells (that produce lactate) and aerobic oxidative tumor cells (that consume lactate) was demonstrated, and MCTs have been discovered to play an important role in this cycle (Figure 12) [250]. So, in glycolytic/hypoxic cancer cells glucose is taken up by GLUT1 and lactate is effluxed by MCT4, and distinctly, in oxidative/oxygenated cancer cells lactate is taken up by MCT1 (Figure 12) [250]. This generates a micro-system, resembling the Cori cycle between liver and muscle, which allows recycling products of anaerobic metabolism to sustain cancer cell energetics.

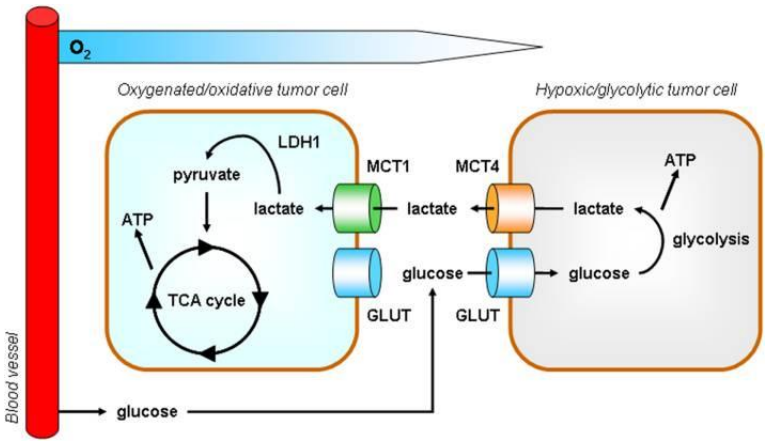

Figure 12. Model according to which tumors behave as metabolic symbionts. Tumor heterogeneity includes metabolism. At a remote location from perfused blood vessels, hypoxic tumors cells rely on glycolysis for survival and proliferation. High ATP production mandatorily depends on high glucose availability and is associated with the release of lactate, a process facilitated by monocarboxylate transporter 4 (MCT4). In contrast, although they also express glucose transporters (GLUT), oxygenated tumor cells have a metabolic preference for lactate versus glucose. MCT1 is a transporter adapted for lactate uptake [250]. This metabolic cooperativity is a key for tumor cell survival under hypoxia in vivo (taken from [234]).

Thus, another strategy for targeting tumoral cell metabolism is disruption of lactate transporters, and in this context MCTs should be considered a promising target for anticancer strategy [250-252]. Importantly, promising results using *in vitro* and *in vivo* models have been reported, where administration of an MCTs inhibitor retarded tumor growth and rendered tumor cells sensitive to radiation [250], and where MCT4 silencing (with RNAi) impaired secretion of lactate generated through glycolysis and induced cell cycle arrest and apoptosis [253].

AIMS

The main aims of the project were to clarify the mechanisms involved in the intestinal transport of BT, their regulation by a variety of xeno and endobiotics, and to search for implications of these processes in colonic health, specifically in CRC. To accomplish this, the specific aims of this study were:

CHAPTER A - Characterization of BT transport at the intestinal epithelial level

- to characterize the uptake of ^{14}C-BT by the human tumoral intestinal epithelial (Caco-2) cell line, and to test the effect of a series of compounds upon it;

- to characterize the uptake of ^{14}C-BT by nontransformed intestinal epithelial cell lines (fetal human colonic epithelial cell line (FHC) and rat intestinal epithelial cell line (IEC-6)), and to test the effect of a series of compounds upon it;

- to investigate the possibility of ^{14}C-BT being transported by the ATP-binding cassette (ABC) transporters P-glycoprotein (MDR1), multidrug resistance proteins (MRPs) or breast cancer resistance protein (BCRP);

CHAPTER B - Modulation of BT transport and of its anticarcinogenic effect

- to investigate the effect of some mineral waters and of some of their constituents upon uptake of ^{14}C-BT by the tumoral intestinal epithelial (Caco-2) cell line;

- to investigate the effect of several dietary polyphenols upon uptake of ^{14}C-BT by the tumoral intestinal epithelial (Caco-2) cell line and to correlate this effect with the modulation of the anticarcinogenic effect of BT in these cells;

- to investigate the effect of *n*-3 polyunsaturated fatty acids (PUFAs), *n*-6 PUFAs, conjugated linoleic acid (CLA) and bile salts upon uptake of ^{14}C-BT by tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines;

- to investigate the effect of the primary bile acid chenodeoxycholic acid (CDCA) upon uptake of ^{14}C-BT by tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines and to correlate this effect with the modulation of the anticarcinogenic effect of BT in these cells;

- to investigate the effect of oxidative stress upon uptake of ^{14}C-BT by tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines;

CHAPTER C - Cancer cell metabolism

- to investigate the effect of cannabinoids upon ^{3}H-2-deoxy-D-glucose (^{3}H-DG) uptake by the tumoral intestinal epithelial (Caco-2) cell line;

- to investigate the effect of clotrimazole in a tumoral intestinal epithelial (Caco-2) cell line, to compare it with the effect in a non-tumoral intestinal epithelial cell line (IEC-6 cells), and to investigate inhibition of energy substrate uptake as a mechanism contributing to its anticarcinogenic effect.

Characterization of BT transport at the intestinal epithelial level

I- Modulation of butyrate transport in Caco-2 cells

Naunyn Schmiedebergs Arch Pharmacol. 2009;379(4):325-36

(doi: 10.1007/s00210-008-0372-x)

IF: 2.500

II- Characterization of butyrate uptake by nontransformed intestinal epithelial cell lines

J Membr Biol. 2011;240(1):35-46

(doi: 10.1007/s00232-011-9340-3)

IF: 1.630

III - The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein

Am J Physiol Cell Physiol. 2011;301(5):C984-94

(doi: 10.1152/ajpcell.00146.2011)

IF: 3.817

Comment in: Am J Physiol Cell Physiol. 2011;301(5):C977-79

I - Modulation of butyrate transport in Caco-2 cells

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DOI 10.1007/s00210-008-0372-x

ORIGINAL ARTICLE

Modulation of butyrate transport in Caco-2 cells

Pedro Gonçalves · João Ricardo Araújo · Maria João Pinho · Fátima Martel

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Abstract The aim of this study was to investigate the putative influence of some pharmacological agents and drugs of abuse upon the apical uptake of butyrate (BT) into Caco-2 cells. The apical uptake of ^{14}C-BT by Caco-2 cells was (1) time and concentration dependent, (2) pH dependent, (3) Na^+ independent and Cl^- dependent, (4) energy dependent, (5) inhibited by several BT structural analogues (acetate, propionate, α-ketobutyrate, pyruvate, lactate), (6) insensitive to the anion exchange inhibitors DIDS and SITS and (7) inhibited by the monocarboxylate transport (MCT) inhibitors NPPB and pCMB. These characteristics are compatible with an involvement of MCT1-mediated transport. Acutely, uptake of a low concentration of ^{14}C-BT (10 μM) was reduced by acetaldehyde, acetylsalicylic acid, indomethacin, caffeine and theophylline and increased by MDMA. Chronically, uptake was increased by caffeine and decreased by tetrahydrocannabinol and MDMA; reverse transcription quantitative real-time PCR analysis showed that these three compounds decreased the mRNA levels of MCT1. Acutely, acetaldehyde, indomethacin and MDMA reduced the uptake of a high concentration of ^{14}C-BT (20 mM), and acetylsalicylic acid increased it. Chronically, none of the compounds affected uptake. Acetaldehyde, indomethacin and propionate seem to be competitive inhibitors of ^{14}C-BT uptake. Acetylsalicylic acid simultaneously increased the K_m and the V_{max} of ^{14}C-BT uptake. In conclusion, MCT1-mediated transport of ^{14}C-BT in Caco-2 cells is modulated by either acute or chronic exposure to some pharmacological agents and drugs of abuse (acetaldehyde, acetylsalicylic acid, indomethacin, caffeine, theophylline and the drugs of abuse tetrahydrocannabinol and MDMA).

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Keywords Caco-2 cells · Butyrate · Uptake · Modulation

Introduction

Short-chain fatty acids (SCFA; acetate, propionate and butyrate) are main end-products of anaerobic bacterial fermentation of dietary fibre within the human colon. Among SCFA, butyrate (BT) exerts potent effects on a variety of colonic mucosal functions and plays a key role in colonic epithelium homeostasis.

One of the proposed beneficial effects of BT on human intestinal health is the prevention and inhibition of colon carcinogenesis. Most of the epidemiological studies showed an inverse relationship between dietary fibre intake and the incidence of colorectal cancer (Bingham et al. 2003; Burkitt 1971; Cassidy et al. 1994; Howe et al. 1992; Kim 2000; Park et al. 2005; Roda et al. 2007; Trock et al. 1990; Vernia et al. 2000). Moreover, exposure of many colon tumour cell lines to BT leads to anticarcinogenic effects by induction of cell differentiation and apoptosis and by inhibition of proliferation, thus reducing the growth rate of colorectal cancer cells (e.g. Hague et al. 1995; Heerdt et al. 1994; Medina et al. 1997; Whitehead et al. 1986). Interestingly enough, the effects of BT on noncarcinogenic cells have been reported as contrary to the effects observed in tumour cell lines as to proliferation, differentiation and apoptosis (e.g. Gibson et al. 1992; Kripke et al. 1989; Sakata and von Engelhardt 1983). This has been referred as the "butyrate paradox".

As SCFA are weak acids ($pK_a \cong 4.8$), more than 90% exist in the anionic, ionised form in the colonic lumen.

Several studies indicate that SCFAs are transported into colonic epithelial cells by two different mechanisms: passive non-ionic diffusion (unspecific component) of the undissociated SCFA form and specific carrier-mediated transport of SCFA anions. Carrier-mediated transport of the ionised form of BT into colonic epithelial cells seems to include (1) a SCFA/HCO_3^- exchanger, (2) an electroneutral H^+-coupled monocarboxylate transporter (MCT) and (3) an electrogenic Na^+-coupled transporter for monocarboxylates (SMCT1). Transport of BT via the SCFA/HCO_3^- exchanger is independent of the Cl^-/HCO_3^- exchanger and of the presence of Na^+ (Harig et al. 1996; Kawamata et al. 2007; Mascolo et al. 1991; McNeil et al. 1979). Transport of BT via the monocarboxylate transporter isoform 1 (MCT1; SLC16A1) is coupled to a transmembrane H^+-gradient (Cuff et al. 2005; Hadjiagapiou et al. 2000; Lecona et al. 2008; Ritzhaupt et al. 1998; Stein et al. 2000). Finally, transport of BT via the Na^+-coupled co-transporter (also denoted Na^+-coupled monocarboxylate transporter (SMCT1; SLC5A8)) is Na^+-dependent (reviewed by Gupta et al. 2006).

The central role of BT in cellular metabolism and the maintenance of colonic tissue homoeostasis make understanding of the regulation of its transport particularly important (reviewed by Cuff and Shirazi-Beechey 2004). In this context, it is very interesting to verify that both MCT1 (Cuff et al. 2005; Daly et al. 2005; Elbashir et al. 2001; Lambert et al. 2002) and SMCT1 (Ganapathy et al. 2005; Gupta et al. 2006) were recently proposed to function as tumour suppressors, the ability of these transporters to mediate the entry of BT into colonic epithelial cells underlying their potential tumour suppressor function. Also, down-regulation of MCT1 seems to be involved in BT deficiency during intestinal inflammation (Thibault et al. 2007). So, both SMCT- and MCT1-mediated transport of BT into colonocytes is fundamental for the regulation of cell homeostasis.

The causes of colorectal carcinoma are multifactorial. Numerous lines of epidemiologic evidence support an association between dietary and other life style factors and the risk of colorectal cancer (Ahmed 2004). So, the aim of this study was to investigate the putative influence of some drugs upon the luminal uptake of BT into colonic epithelial cells. For this, we characterised the apical uptake of ^{14}C-BT by Caco-2 cells, and we tested the acute and chronic effect of these compounds upon it. The human colonic adenocarcinoma Caco-2 cell line forms monolayers of viable and polarised intestinal epithelial cells and mimics the physiological functions of small intestinal absorptive cells, exhibiting several functional properties, such as carrier-mediated transport systems and membrane enzyme activities, of intestinal epithelial cells (reviewed by Delie and Rubas 1997).

Materials and methods

Caco-2 cell culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage numbers 37 and 67. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 5.55 mM glucose and supplemented with 15% foetal calf serum, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2 to 3 days, and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3 and sub-cultured in plastic culture dishes (21 cm^2; ∅ 60 mm; Corning Costar, Corning, NY, USA). For uptake studies, Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar), and the experiments were performed 7–11 days after the initial seeding. The cell medium was free of foetal calf serum for 24 h before the experiments.

Determination of ^{14}C-BT uptake by Caco-2 cells

The uptake experiments were performed with Caco-2 cells incubated in glucose-free Krebs buffer (containing, in millimolars, 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4 and either 20 HEPES (pH 7.5 and 8.0) or 20 MES (pH 5.5 and 6.5). Initially, the culture medium was aspirated, and the cells were washed with 0.3 ml buffer at 37°C. Then, the cell monolayers were pre-incubated for 20 min in 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.3 ml medium at 37°C containing 10 μM or 20 mM ^{14}C-BT (except in the experiments for determination of the kinetics of uptake). Incubation was stopped after 3 min (except in the time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.3 ml ice-cold buffer. The cells were then solubilised with 0.3 ml 0.1% (*v*/*v*) Triton X-100 (in 5 mM Tris–HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Acute and chronic treatment of the cells

The concentrations of compounds to test both acutely and chronically were chosen based on previous works from our group (Araújo et al. 2008; Keating et al. 2006,2008; Lemos et al. 2007, Monteiro et al. 2005).

The acute effect of compounds on ^{14}C-BT uptake was tested by pre-incubating (20 min) and incubating cells with

^{14}C-BT (3 min) in the presence of the compounds to be tested.

The chronic effect of compounds on ^{14}C-BT uptake was tested by cultivating 3-day-old cell cultures (90–95% confluence) in culture medium in the presence of the compounds to be tested. The medium was renewed daily, and the transport experiments were performed after 48 h. The transport experiments were identical to the experiments described above, except that there was no pre-incubation period, and cells were incubated with ^{14}C-BT (3 min) in the absence of drugs.

Assessment of cell viability

The effect of compounds on Caco-2 cell viability was determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay (Mosmann 1983).

To test whether the compounds that had an acute effect (23 min) upon ^{14}C-BT uptake affected cellular viability, cells were incubated for 3 h at 37°C in 500 µl of culture medium with 0.5 mg/ml MTT solution. In the last 23 min of this period, the compounds to be tested were added.

To test whether the compounds that had a chronic effect (48 h) upon ^{14}C-BT uptake affected cellular viability, cells were chronically treated with the compounds as described above. After 45 h of treatment, 50 µl MTT solution (5 mg/ml) was added to each well. The cells were then further incubated for 3 h at 37°C.

The MTT solution was removed after the 3-h incubation period, and the cells were lysed by addition of 200 µl DMSO followed by plate shaking for 10 min at room temperature. Optical density for the solutions in each well was determined at both 550 and 650 nm. Optical density at 650 nm corresponds to unspecific light absorption and was subtracted from the OD at 550 nm to give the OD value specific to formazan crystals derived from MTT cleavage.

Protein determination

The protein content of cell monolayers was determined as described by Bradford (1976) using human serum albumin as standard.

Reverse transcription quantitative real-time PCR

Total RNA was extracted from chronically treated Caco-2 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Germany).

Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen Corporation, CA, USA) according to manufacturer's instructions, and 0.5 µg of resulting DNA-free RNA was reverse-transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 20 µl of final reaction volume, according to manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade unreacted RNA. For the quantitative real-time PCR, 2 µl of the 20 µl reverse transcription reaction mixture was used. For the calibration curve, Caco-2 standard cDNA was diluted in five different concentrations.

The primer pair used for amplification/quantification of human MCT1 was as follows: 5′-CAC CGT ACA GCA ACT ATA CG-3′ (forward) and 5′-CAA TGG TCG CCT CTT GTA GA-3′ (reverse; as previously described by Maubon et al. 2007); the primer pair for human β-actin was kindly donated by Dr. Joana Marques (Department of Genetics, Faculty of Medicine, University of Porto, Portugal).

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ, USA). Reactions (20 µl) were set up in microcapillary tubes using 0.5 µM of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification (95°C for 10 s, annealing temperature (AT) for 10 s and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10-s segment) repeated 40 times, a melting curve program ((AT+10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C. The ATs were 60°C and 65°C for MCT1 and β-actin genes, respectively. Data were analysed using LightCycler analysis software.

Calculation and statistics

For the analysis of the time course of ^{14}C-BT uptake, the parameters of Eq. 1 were fitted to the experimental data by a non-linear regression analysis using a computer-assisted method (Muzyka et al. 2005).

$$A(t) = k_{in}/k_{out}\left(1 - e^{-k_{out}\times t}\right) \quad (1)$$

$A(t)$ represents the accumulation of ^{14}C-BT at time t, k_{in} and k_{out} are the rate constants for inward and outward transport, respectively, and t is the incubation time. A_{max} corresponds to the accumulation ($A(t)$) at steady state ($t\rightarrow\infty$). K_{in} is given in picomoles per milligram protein minute and k_{out} in minute^{-1}. In order to obtain clearance values, k_{in} was converted to microlitres per milligram protein minute.

For the analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis–Menten equation were fitted to the experimental data by a non-linear regression analysis using a computer-assisted method (Muzyka et al. 2005).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. Statistical

significance of the difference between various groups was evaluated by the Student's *t* test. Differences were considered to be significant when $P<0.05$.

Materials

The following materials were used: ^{14}C-BT (*n*-butyric acid, sodium salt, [1-^{14}C]; specific activity 30–60 mCi/mmol (Biotrend Chemikalien GmbH, Köln, Germany); acetylsalicylic acid, acetic acid sodium salt, alpha-cyano-4-hydroxycinnamic acid, choline chloride, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt), dinitrophenol, ethanol, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 4-(hydroxymercuri) benzoic acid sodium salt, indomethacin, ketobutyric acid sodium salt hydrate, L-lactic acid sodium salt, lithium chloride, MES (2-[*N*-morpholino]ethanesulfonic acid) hydrate), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide), (−)nicotine hydrogen tartrate, 5-nitro-2-(3-phenylpropylamino)benzoic acid, propionic acid sodium salt, pyruvic acid sodium salt, SITS (4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid dissodium salt hydrate), sodium azide, sodium fluoride, succinic acid sodium salt, theophylline, trypsin-EDTA solution (Sigma, St. Louis, MO, USA); DMSO (dimethylsulfoxide), Triton X-100 (Merck, Darmstadt, Germany); foetal calf serum (Invitrogen Corporation, Carlsbad, CA, USA); (±)-amphetamine, (±)-MDMA (ecstasy; 3,4-methylenedioxymetamphetamine), THC ((−)-Δ^9-tetrahydrocannabinol (tetrahydro-6,6,9-trimethyl-3-pentyl-6*H*-dibenzo[*b*,*d*]pyran-1-ol)) (Cerilliant Corporation, Round Rock, TX, USA); acetaldehyde (May & Baker, Dagenham, UK); caffeine (BDH Laboratory Chemicals Ltd., Poole, UK); cocaine hydrochloride (Uquipa, Lisbon, Portugal).

The drugs to be tested were dissolved in water, ethanol, methanol or DMSO; the final concentration of these solvents being 1% in the buffer or 0.1% in the culture media for acute or chronic treatments, respectively. Controls for these drugs were run in the presence of the solvent.

Results

Time and pH dependence of ^{14}C-BT apical uptake in Caco-2 cells

In a first series of experiments, we determined the time course of ^{14}C-BT apical uptake by Caco-2 cells. For this, cells were incubated with two distinct concentrations of ^{14}C-BT (10 or 100 μM) for various periods of time.

As shown in Fig. 1, Caco-2 cells accumulated 10 or 100 μM ^{14}C-BT in a time-dependent way. Uptake of 10 μM ^{14}C-BT was found to be comparatively higher than uptake at 100 μM. Moreover, there seems to exist some binding of ^{14}C-BT to the membrane when uptake of 100 μM of this compound was studied (Fig. 1b). Finally, uptake of both 10 and 100 μM ^{14}C-BT was found to be pH dependent, increasing as the pH decreases from 8 to 5.5 (Figs. 1 and 2a).

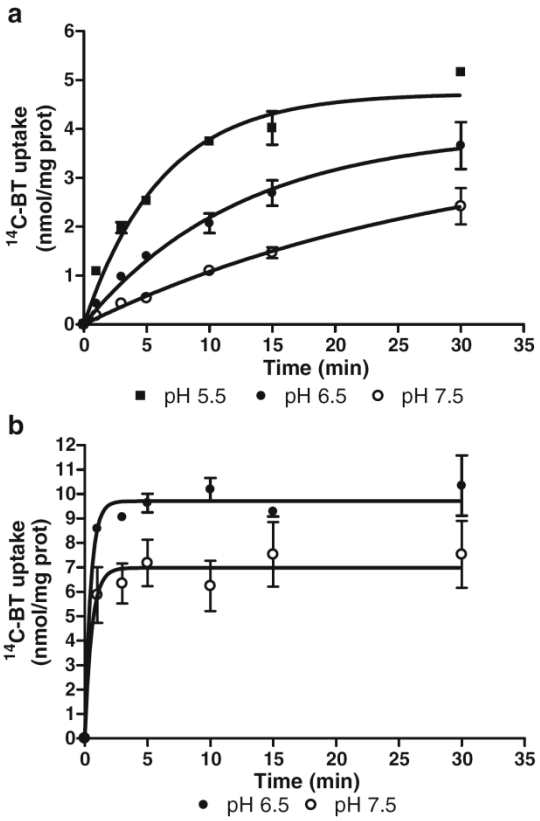

Fig. 1 Time course of ^{14}C-BT apical uptake in Caco-2 cells. Caco-2 cells were incubated at 37°C with **a** 10 μM (n=4) or **b** 100 μM (n=2–4) ^{14}C-BT. Shown are arithmetic means ± SEM

So, in subsequent experiments, cells were exposed to 10 μM ^{14}C-BT at a pH of 6.5 in order to characterise uptake of this compound. Because uptake of 10 μM ^{14}C-BT at pH 6.5 was linear with time for up to 3 min of incubation, a 3-min incubation time was selected as the standard incubation time in subsequent experiments in order to measure initial rates of uptake.

Ionic dependence of ^{14}C-BT apical uptake in Caco-2 cells

To test for the dependence of the apical uptake of ^{14}C-BT on extracellular Na^+ and Cl^-, we measured uptake in the absence of NaCl, which was substituted by either LiCl,

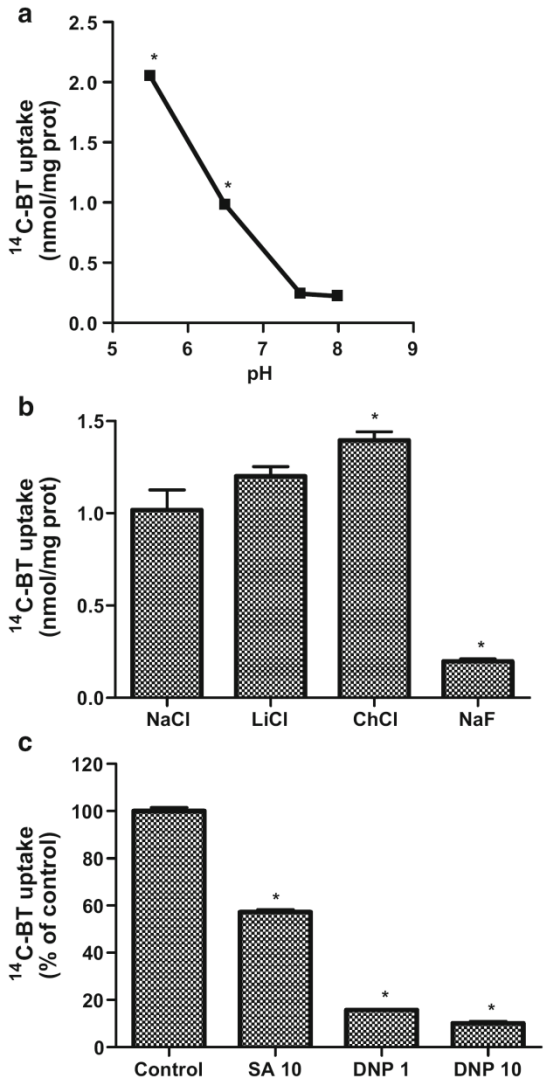

Fig. 2 Characteristics of ^{14}C-BT apical uptake in Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min. **a** pH dependence. The extracellular pH in the pre-incubation and incubation media ranged from 5.5 to 8.0 (n=6). *Significantly different from uptake at pH 7.5; **b** ionic dependence. NaCl in the in the pre-incubation and incubation media was isotonically replaced by either LiCl, choline chloride (*ChCl*) or NaF (n=6). *Significantly different from control (*NaCl*); **c** energy dependence. Caco-2 cells were incubated under control conditions (n=6–7) or in the presence of sodium azide 10 mM (*SA 10*; n=6) or dinitrophenol 1 mM (*DNP 1*; n=4) or 10 mM (*DNP 10*; n=5). *Significantly different from control. Shown are arithmetic means ± SEM

choline chloride or NaF. As shown in Fig. 2b, substitution of Cl^- with F^- caused a dramatic decrease in the uptake of ^{14}C-BT. On the other hand, substitution of Na^+ with Li^+ had no effect on the uptake of ^{14}C-BT. Finally, a small but significant increase in ^{14}C-BT uptake was observed when Na^+ was substituted with choline.

Energy dependence of ^{14}C-BT apical uptake in Caco-2 cells

Uptake of ^{14}C-BT by Caco-2 cells was found to be highly energy dependent, as it was greatly reduced in the presence of either sodium azide (10 mM) or dinitrophenol (1 or 10 mM; Fig. 2c).

Kinetics of ^{14}C-BT apical uptake in Caco-2 cells

The relationship between the initial rates of uptake of ^{14}C-BT and its concentration in the apical medium (from 10 μM to 3 mM) is represented in Fig. 3. They were analysed according to the Michaelis–Menten equation (see "Materials and methods"). The evaluated kinetic parameters V_{max} and K_m were 86.8±6.6 nmol/mg prot/3 min and 2.83±0.38 mM, respectively (Table 1).

Pharmacological characterisation of ^{14}C-BT apical uptake in Caco-2 cells

Effect of structural analogues

The effect of acetate and propionate (which, together with butyrate, constitute the major SCFA present in the lumen of the colon) and other monocarboxylates (L-lactate, pyruvate, α-ketobutyrate) on the initial rates of ^{14}C-BT uptake was determined. As shown in Fig. 4a, ^{14}C-BT uptake was significantly reduced by all the monocarboxylates tested. On the other hand, the dicarboxylate succinate showed no effect.

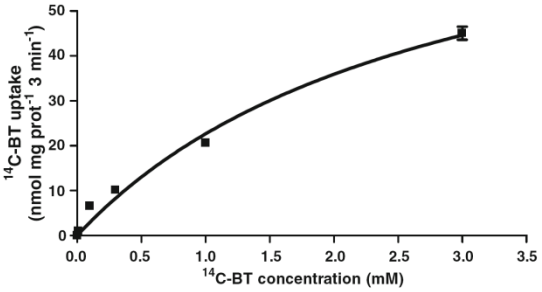

Fig. 3 Kinetics of ^{14}C-BT apical uptake in Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 0.01–3 mM ^{14}C-BT for 3 min under control conditions (n=27). Shown are arithmetic means ± SEM

Table 1 Kinetic parameters of ^{14}C-BT apical uptake and effect of different compounds

	V_{max} (nmol/mg prot/3 min)	K_m (mM)	Samples
Control	86.8±6.6	2.83±0.38	27
PROP	106.2±17.2	5.83±1.35[a]	6
ACA	113.6±35.6	6.59±2.86[a]	6
ASA	151.2±41.27[a]	5.94±2.31[a]	10
IND	154.4±82.9	14.8±9.4[a]	6

Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 0.01–3 mM ^{14}C-BT for 3 min, under control conditions or in the presence of propionate 10 mM (PROP), acetaldehyde 30 mM (ACA), acetylsalicylic acid 5 mM (ASA) or indomethacin 1 mM (IND). Shown are arithmetic means ± SEM

[a] Significantly different from control

Effect of potential inhibitors

We also tested the effect of the classical anion exchange inhibitors, DIDS and SITS, upon ^{14}C-BT uptake. None of these compounds was able to affect the uptake of ^{14}C-BT; instead, uptake was even slightly increased in the presence of these compounds (Fig. 4b). Moreover, we also tested the effect of several monocarboxylate transporter (MCT1) inhibitors. With the exception of 4-CHC, all the tested MCT1 inhibitors reduced the uptake of ^{14}C-BT significantly (>40%), with NPPB showing the greatest inhibition (80%).

Modulation of ^{14}C-BT apical uptake in Caco-2 cells by drugs

In this series of experiments, the acute and chronic effects of several pharmacological agents and abuse substances upon the apical uptake of ^{14}C-BT by Caco-2 cells were investigated.

Effect upon the apical uptake of a low concentration (10 μM) of ^{14}C-BT

Acute effect As shown in Fig. 5, nicotine (0.1–100 μM), cocaine (0.1–10 μM), THC (1–100 nM) and amphetamine (0.1–10 μM) were devoid of effect upon the uptake of ^{14}C-BT. Ethanol also showed no effect upon ^{14}C-BT uptake, but its metabolite acetaldehyde reduced ^{14}C-BT uptake in a concentration-dependent manner, to a maximum of 32% of control. Caffeine (1 μM) and theophylline (10–100 μM) caused a small (10–20%) but significant reduction in ^{14}C-BT uptake. Moreover, acetylsalicylic acid (5 mM) and indomethacin (1 mM) reduced ^{14}C-BT uptake by 30% and 55%, respectively. Finally, MDMA (0.1 μM) caused a small (5–10%) but significant increase in the uptake of this compound (Fig. 5).

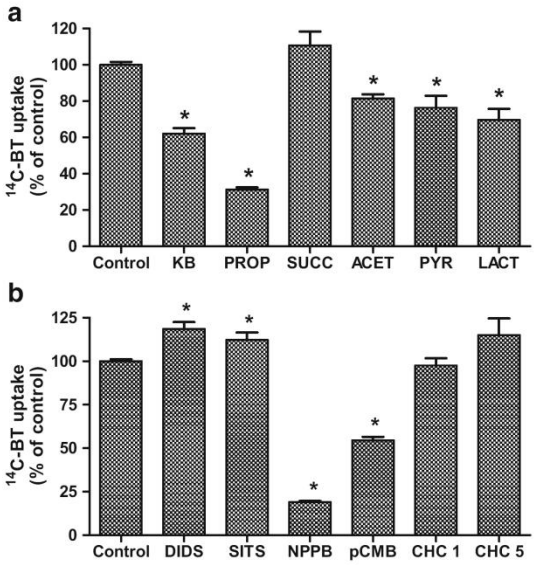

Fig. 4 Effect of butyrate structural analogues (**a**) and potential inhibitors (**b**) on ^{14}C-BT apical uptake in Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min under control conditions (n=12–17) or in the presence of **a** α-ketobutyrate 10 mM (*KB*; n=6), propionate 10 mM (*PROP*; n=6), succinate 10 mM (*SUCC*; n=9), acetate 10 mM (*ACET*; n=6), pyruvate 10 mM (*PYR*; n=9) or L-lactate 10 mM (*LACT*; n=6) or **b** DIDS 0.5 mM (*DIDS*; n=6), SITS 0.5 mM (*SITS*; n=6), 5-nitro-2-(3-phenylpropylamino)benzoate 0.5 mM (*NPPB*; n=7), *p*-chloromercuribenzoate 0.5 mM (*pCMB*; n=7) or α-cyano-4-hydroxycinnamic acid 1 mM (*CHC 1*; n=10) or 5 mM (*CHC 5*; n=7). Shown are arithmetic means ± SEM. *Significantly different from control

Chronic effect Nicotine (0.1–10 μM), cocaine (0.01–1 μM), ethanol (0.1–3 mM), acetaldehyde (0.1–1 mM), acetylsalicylic acid (0.01–0.5 mM), indomethacin (1–100 μM), theophylline (0.1–10 μM) and amphetamine (0.1–2.5 μM) did not change the uptake of ^{14}C-BT by the cells. However, caffeine (10 μM) caused a 10% increase in uptake, and THC (1 and 100 nM) and MDMA (0.1–1 μM) caused a 15% decrease in the uptake of ^{14}C-BT (Fig. 6).

Effect upon the apical uptake of a high concentration (20 mM) of ^{14}C-BT

We next examined the effect of the compounds, which were shown to affect the uptake of a low concentration of ^{14}C-BT upon the uptake of a higher (20 mM) concentration of this substrate.

Acute effect The effect of acetaldehyde, caffeine, theophylline, acetylsalicylic acid, indomethacin and MDMA were tested (Fig. 7a). Of these, acetaldehyde (100 mM), indo-

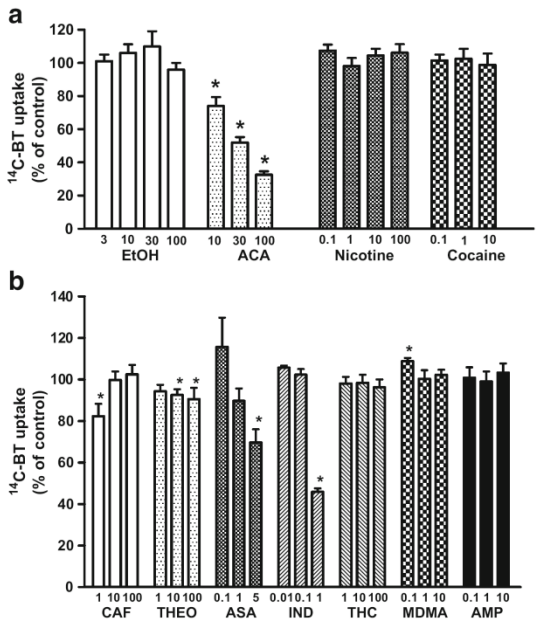

Fig. 5 Acute effect of several drugs upon the uptake of ^{14}C-BT (10 μM) in Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min, in the absence (*control*) or in the presence of **a** ethanol (*EtOH*; 3–100 mM; *n*=9), acetaldehyde (*ACA*; 10–100 mM; *n*=9), nicotine (0.1–100 μM; *n*=6–9), and cocaine (0.1–10 μM; *n*=9), **b** caffeine (*CAF*; 1–100 μM; *n*=9), theophylline (*THEO*; 1–100 μM; *n*=6), acetylsalicylic acid (*ASA*; 0.1–5 mM; *n*=9), indomethacin (*IND*; 10–1000 μM; *n*=6–7), Δ^9-tetrahydrocannabinol (*THC*; 1–100 nM; *n*=6), MDMA (0.1–10 μM; *n*=6) and amphetamine (*AMP*; 0.1–10 μM; *n*=9). Shown are arithmetic means ± SEM. *Significantly different from control

methacin (1 mM) and MDMA (0.1 μM) were found to significantly reduce uptake of ^{14}C-BT. On the contrary, acetylsalicylic acid (5 mM) produced a significant increase in the uptake of this compound (Fig. 7).

Chronic effect We tested the effect of caffeine, THC and MDMA. As shown in Fig. 7b, none of these compounds produced a significant change in the uptake of ^{14}C-BT by Caco-2 cells.

Effect of the drugs upon the kinetic parameters of ^{14}C-BT apical uptake

We next tested the effect of some compounds upon the kinetic parameters of ^{14}C-BT apical uptake. The compounds tested were those that, acutely, showed a significant effect upon the uptake of a low concentration (10 μM) of ^{14}C-BT (see Fig. 5). As can be seen in Table 1, acetaldehyde and indomethacin seem to behave as competitive inhibitors of ^{14}C-BT uptake. On the other hand, acetylsalicylic acid showed a very unusual effect: this compound simultaneously increased the K_m and the V_{max} of ^{14}C-BT uptake. So, acetylsalicylic acid seems to cause a decrease in the affinity of ^{14}C-BT uptake while increasing the capacity of the uptake. Propionate was also tested, and this compound also behaved as a competitive inhibitor of ^{14}C-BT uptake (Table 1).

Effect of the drugs upon cell viability

We also investigated the effect of the compounds, which affected ^{14}C-BT uptake upon the viability (expressed as the amount of MTT cleaved per milligram protein) of the Caco-2 cells.

Acute effect Acetaldehyde (10–100 mM), MDMA (0.1 μM), caffeine (1 μM), theophylline (100 μM) and acetylsalicylic acid (5 mM) did not affect cell viability (results not shown). However, cellular viability increased in the presence of indomethacin (1 mM; to 121.5±2.8% of control; *n*=6).

Chronic effect THC (100 nM) and MDMA (1 μM) did not affect cellular viability (results not shown). On the other hand, caffeine (10 μM) significantly reduced it (to 73.5± 3.5% of control; *n*=6).

Reverse transcription quantitative real-time PCR

In order to investigate the hypothesis that the chronic effect of caffeine, THC and MDMA upon the apical uptake of a low concentration (10 μM) of ^{14}C-BT (Fig. 6b) is related to changes in MCT1 gene expression levels, we quantified MCT1 mRNA in control Caco-2 cells and in chronically treated cells by reverse transcription quantitative real-time PCR. As shown in Fig. 8, treatment of the cells with all the tested compounds produced a significant decrease (by 20–30%) in MCT1 gene expression levels.

Discussion

Intestinal health and function are strongly dependent on appropriate levels of SCFA. Among the SCFA, BT is especially important for colonocyte health and integrity. This is because BT is known to have multiple regulatory roles in the mammalian colon, including (1) being the main energy source for the colonocyte (Roediger 1980, 1982; Scheppach et al. 1992a, b), (2) inhibiting colon carcinogenesis (it suppresses growth of cancer cells by inducing differentiation and apoptosis and by inhibiting cell prolif-

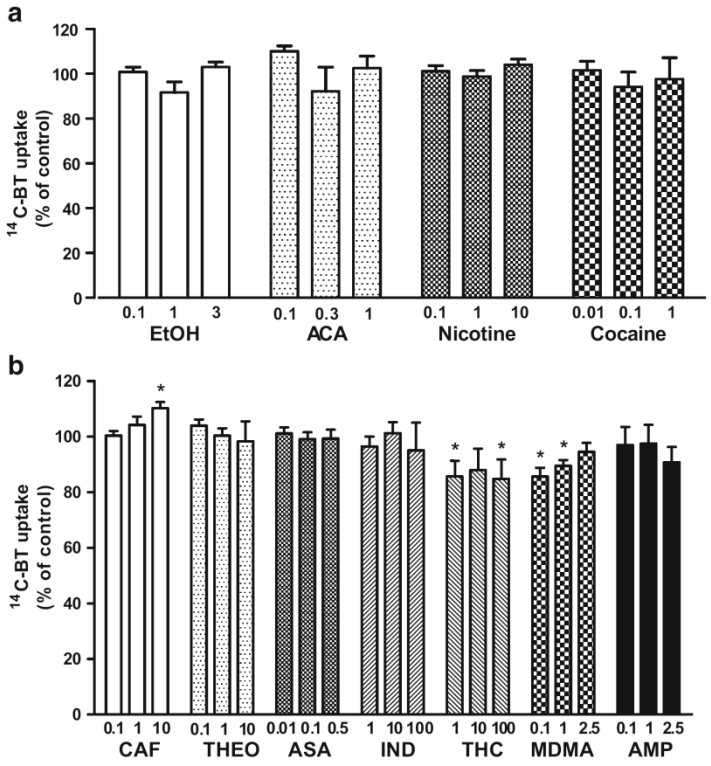

Fig. 6 Chronic effect of several compounds upon the uptake of ^{14}C-BT (10 μM) by Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min, after being cultivated for 48-h in the absence (*control*) or in the presence of **a** ethanol (*EtOH*; 0.1–3 mM; n=9–12), acetaldehyde (*ACA*; 0.1–1 mM; n=6–9), nicotine (0.1–10 μM; n=6) and cocaine (0.01–1 μM; n=5–6), **b** caffeine (*CAF*; 0.1–10 μM; n=6), theophylline (*THEO*; 0.1–10 μM; n=6), acetylsalicylic acid (*ASA*; 0.01–0.5 mM; n=6), indomethacin (*IND*; 1–100 μM; n=12), Δ^9-tetrahydrocannabinol (*THC*; 1–100 nM; n=9), MDMA (0.1–2.5 μM; n=9–12) and amphetamine (*AMP*; 0.1–2.5 μM; n=12). Shown are arithmetic means ± SEM. *Significantly different from control

eration; e.g. Hague et al. 1995; Heerdt et al. 1994; Medina et al. 1997; Whitehead et al. 1986), (3) promoting growth and proliferation of normal colonic epithelial cells (e.g. Gibson et al. 1992; Kripke et al. 1989; Sakata and von Engelhardt 1983), (4) stimulating fluid and electrolyte absorption (Binder and Mehta 1989; Montrose and Kere 2001; Resta-Lenert et al. 2001), (5) inhibiting colon inflammation and oxidative stress and (6) improving the colonic defence barrier function (reviewed by Hamer et al. 2008; Wong et al. 2006).

Given the central role of BT in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important (reviewed by Cuff and Shirazi-Beechey 2004). However, very little is known concerning this issue: MCT1-mediated transport at the intestinal level is up-regulated by its substrate, BT (Borthakur et al. 2008; Cuff et al. 2002), enhanced by leptin (Buyse et al. 2002) and phorbol 12-myristate 13-acetate (Alrefai et al. 2004) and inhibited by enteropathogenic *Escherichia coli* (Borthakur et al. 2006), interferon-γ and tumour necrosis factor-α (Thibault et al. 2007). So, we decided to investigate the effect of some pharmacological agents and some drugs of abuse upon this mechanism.

The apical uptake of ^{14}C-BT by Caco-2 cells was found to be (1) time and concentration dependent, (2) pH dependent, with uptake increasing with decreasing pH, (3) Na^+ independent and Cl^- dependent, (4) energy dependent, (5) inhibited by several structural analogues (acetate, propionate, α-ketobutyrate, pyruvate, lactate), (6) insensitive to the anion exchange inhibitors DIDS and SITS and (7) inhibited by the monocarboxylate transport inhibitors NPPB and pCMB, but not by 4-CHC.

The apical uptake of BT by Caco-2 cells was previously characterised by other groups (Hadjiagapiou et al. 2000; Lecona et al. 2008; Stein et al. 2000). Interestingly enough, the main features of ^{14}C-BT uptake in our experiments perfectly fits those previously described: the time and the pH dependence of uptake, the Na^+ independence, the saturation kinetics (a K_m of 2.8 mM in the present study and a K_m of 2.4–2.6 mM in the previous ones; Hadjiagapiou et al. 2000; Stein et al. 2000), the lack of inhibition by the anion exchange process inhibitors DIDS and SITS, the inhibition by BT structural analogues (monocarboxylates) but not dicarboxylates and the inhibition by MCT inhibitors (Hadjiagapiou et al. 2000; Stein et al. 2000).

From these results, we conclude that the characteristics of ^{14}C-BT uptake by Caco-2 cells are compatible with an

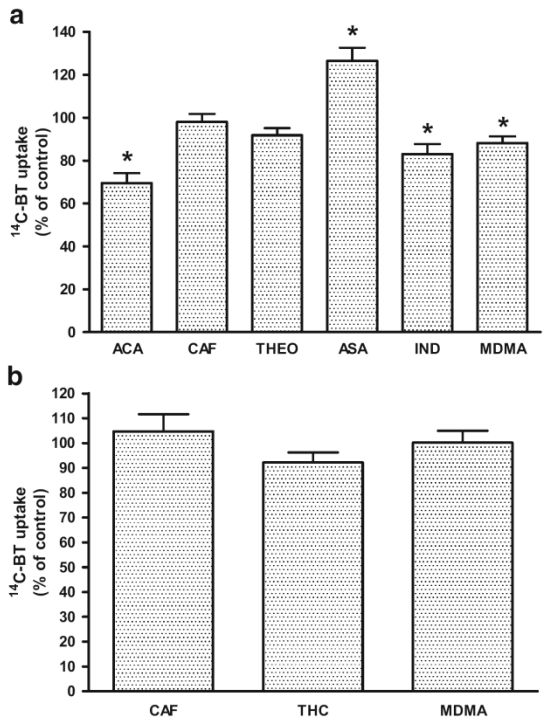

Fig. 7 Acute (**a**) and chronic (**b**) effect of several compounds upon the uptake of ^{14}C-BT (20 mM) by Caco-2 cells. **a** Initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 20 mM ^{14}C-BT for 3 min, in the absence (*control*) or in the presence of acetaldehyde (*ACA*; 100 mM; n=6), caffeine (*CAF*; 1 μM; n=15), theophylline (*THEO*; 100 μM; n=15), acetylsalicylic acid (*ASA*; 5 mM; n=12), indomethacin (*IND*; 1 mM; n=12) or MDMA (0.1 μM; n=9); **b** initial rates of uptake were determined in Caco-2 cells incubated at 37°C with 20 mM ^{14}C-BT for 3 min, after being cultivated for 48-h in the absence (*control*) or in the presence of caffeine (*CAF*; 10 μM; n=12), Δ^9-tetrahydrocannabinol (*THC*; 0.1 μM; n=12) or MDMA (1 μM; n=12). Shown are arithmetic means ± SEM. *Significantly different from control

involvement of MCT1. MCT1 is highly expressed in Caco-2 cells (Hadjiagapiou et al. 2000; Lecona et al. 2008). Interestingly enough, MCT1 was also suggested to play a major role in the apical uptake of BT by Caco-2 cells in the two previous reports (Hadjiagapiou et al. 2000; Stein et al. 2000).

A very recent report describing the characteristics of BT uptake in Caco-2 cells refers the existence of two carrier-mediated mechanisms: a low-affinity/high-capacity mechanism (K_m=109 mM) and a high-affinity/low-capacity mechanism (K_m=18 μM), with distinct characteristics; whereas the low-affinity uptake is inhibited by BT structural analogues, is Na^+ independent, not affected by 4-CHC and increased by DIDS, the high-affinity uptake is inhibited by BT structural analogues, but is Na^+ dependent, inhibited by 4-CHC and not affected by DIDS (Lecona et al. 2008). The characteristics of ^{14}C-BT uptake in our experiments are very similar to those of the low-affinity component of BT uptake described by Lecona et al. (2008).

We next investigated the effect of several pharmacological agents upon the uptake of ^{14}C-BT. The effect of the compounds was analysed both at a low (10 μM) and at a high concentration (20 mM) of ^{14}C-BT. These two concentrations have physiological relevance. On the one hand, a high concentration (in the millimolar range) of BT in the colonic lumen may be attained, e.g. after digestion of dietary fibre (the concentrations of these fatty acids in the lumen may reach 70–130 mM, with a 20–30% BT; Cummings 1981; Mortensen and Clausen 1996). On the other hand, a low concentration (in the micromolar range) of BT may be attained, e.g. in the intermeal period, at the bottom of colonic crypts or in the inner layers of tumours.

Acutely, uptake of a low concentration of ^{14}C-BT (10 μM) was reduced in the presence of acetaldehyde (maximally by 68%), acetylsalicylic acid and indomethacin (by 30% and 55%, respectively) and caffeine and theophylline (by 10–20%). On the other hand, it was increased (by 5–10%) in the presence of MDMA. Chronically, it was increased (by 10%) by caffeine and decreased (by 15%) in the presence of either THC or MDMA.

Acutely, uptake of a high concentration of ^{14}C-BT (20 mM) was reduced by acetaldehyde, indomethacin and MDMA (by 30%, 25% and 12%, respectively) and increased (by 27%) by acetylsalicylic acid. Chronically, none of the compounds affected the uptake of ^{14}C-BT.

The inhibitory or stimulatory effect of the compounds upon the apical uptake of ^{14}C-BT was not related to an effect upon cellular viability. With the exception of acute indomethacin, which increased cellular viability, and chronic caffeine, which decreased it, none of the tested compounds showed a significant effect upon this parameter.

Analysis of the effect of the compounds, which, acutely, affected the apical uptake of a low (10 μM) concentration of ^{14}C-BT, upon the kinetic parameters of uptake suggests that acetaldehyde, indomethacin and propionate are competitive inhibitors of ^{14}C-BT uptake. On the other hand, acetylsalicylic acid simultaneously increased the K_m and the V_{max} of ^{14}C-BT uptake, thus appearing to decrease the affinity of ^{14}C-BT uptake while increasing the transporter capacity. So, this compound might inhibit uptake of a low concentration of ^{14}C-BT (in the micromolar range) while increasing uptake of a high concentration (in the millimolar range) and this was indeed what was verified (see above).

On the other hand, analysis of the effect of the compounds, which, chronically, affected the apical uptake of a low concentration of ^{14}C-BT (10 μM), upon MCT1 gene expression levels revealed that caffeine, THC and

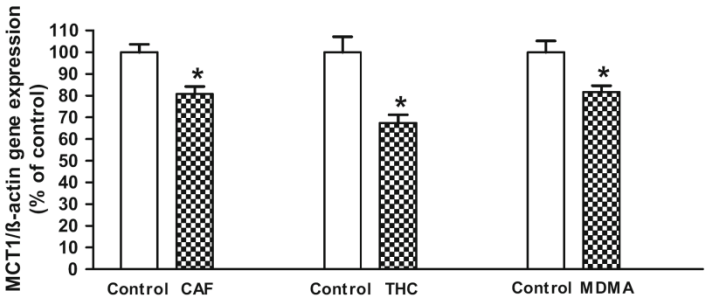

Fig. 8 Gene expression levels of the monocarboxylate transporter isoform 1 (*MCT1*) in Caco-2 cells chronically treated with caffeine (*CAF*; 10 μM; *n*=5), Δ^9-tetrahydrocannabinol (*THC*; 100 nM; *n*=6) or MDMA (1 μM; *n*=6). Caco-2 cells were cultivated for 48 h in the absence (*control*; *n*=5–6) or presence of each of these compounds. MCT1 mRNA levels were quantified by reverse transcription quantitative real-time PCR. Shown are arithmetic means ± SEM corresponding to the expression of MCT1 relative to β-actin. *Significantly different from control

MDMA caused a 20–30% reduction in the mRNA levels of this transporter. These results strongly suggest that, for both THC and MDMA, the decrease in ^{14}C-BT uptake results from a decrease in the expression level of MCT1. However, for caffeine, the small but significant increase in ^{14}C-BT uptake is most probably not related to changes in the expression level of MCT1, as this agent decreased this parameter. Because caffeine exerts multiple effects at the cellular level (e.g. it is a adenosine receptor antagonist and a phosphodiesterase inhibitor), thus affecting the intracellular concentration of second messengers and knowing that MCT1 is subject to regulation by intracellular mechanisms (see above), we hypothesise that the increase in ^{14}C-BT uptake produced by caffeine may result from an increase in the activity of MCT1.

Interestingly enough, most of the compounds found to affect ^{14}C-BT uptake by Caco-2 cells in our experiments are also related with colorectal cancer. The nonsteroidal anti-inflammatory drug (NSAID) inhibitors of cyclooxygenase/lipoxygenase families of enzymes (such as acetylsalicylic acid and indomethacin) emerged as a new perspective in tumour therapy as well as cancer prevention (Chan 2002; Cuendet and Pezzuto 2000; Kawai et al. 2002; Shiff et al. 1996). Similarly, there is emerging evidence that cannabinoids, especially THC, may represent novel anticancer agents due to their ability to regulate signalling pathways critical for cell growth and survival and that the use of THC or selective targeting of the CB1 receptor may represent a novel strategy for colorectal cancer therapy (Greenhough et al. 2007). On the other hand, epidemiological data have identified chronic alcohol consumption as a significant risk factor for colorectal cancer. Among the pathophysiological mechanisms involved in this effect, evidence has accumulated that generation of acetaldehyde is the main responsible for alcohol associated carcinogenesis. Acetaldehyde is highly toxic, mutagenic and carcinogenic (Pöschl and Seitz 2004; Seitz and Homann 2007). From our results, we might speculate that interference with the uptake of BT by the colonic epithelium might contribute to the colorectal cancer suppressor effect of NSAIDs and THC and to the colorectal cancer promotor effect of acetaldehyde.

On the other hand, there is little evidence that coffee or tea consumption increases the risk of cancer (Higdon and Frei 2006; Tavani and La Vecchia 2004); some researchers even contend that a link between high consumption of coffee and a low incidence of colorectal cancer has been firmly established (Michels et al. 2005). Our results, showing that chronic caffeine increased ^{14}C-BT (10 μM) uptake by Caco-2 cells while simultaneously decreasing the cellular viability, are very interesting in this perspective.

To our knowledge, this is the first report describing an inhibitory effect of the NSAIDs acetylsalicylic acid and indomethacin on ^{14}C-BT uptake by Caco-2 cells. It was recently described that ibuprofen and other structurally related NSAIDs interact with hSMCT1 (Coady et al. 2004; Itagaki et al. 2006) and MCTs (Tamai et al. 1995; Choi et al. 2005). Interestingly, these drugs are transportable substrates for MCTs but are non-transportable blockers of SMCT1 (Coady et al. 2004; Choi et al. 2005; Itagaki et al. 2006; Tamai et al. 1995). Based on the analysis of the effect of acetylsalicylic acid and indomethacin upon the kinetic parameters of ^{14}C-BT uptake by Caco-2, we suggest that these two compounds inhibited MCT1-mediated transport.

In conclusion, our results suggest the involvement of MCT1 in the apical uptake of ^{14}C-BT by Caco-2 cells. Moreover, they suggest that MCT1-mediated transport is modulated by either acute or chronic exposure to some pharmacological agents, including drugs of abuse (acetaldehyde, acetylsalicylic acid, indomethacin, caffeine, theophylline, THC and MDMA). Finally, it should be noted that (1) the acute and chronic effect of the compounds upon the uptake of ^{14}C-BT uptake may be distinct and that (2)

the effect of the compounds upon the apical uptake of a low and a high concentration of BT may also be distinct. This last point is very interesting in the context of the known variations in the intraluminal concentration of BT that exist, e.g. between the fasting/feeding period and in some pathological conditions (e.g. colon cancer and ulcerative colitis).

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II- Characterization of butyrate uptake by nontransformed intestinal epithelial cell lines

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Characterization of Butyrate Uptake by Nontransformed Intestinal Epithelial Cell Lines

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Abstract Butyrate (BT) is one of the main end products of anaerobic bacterial fermentation of dietary fiber within the human colon. Among its recognized effects, BT inhibits colon carcinogenesis. Our aim was to characterize uptake of BT by two nontransformed intestinal epithelial cell lines: rat small intestinal epithelial (IEC-6) and fetal human colonic epithelial (FHC) cells. Uptake of ^{14}C-BT by IEC-6 cells was (1) time- and concentration-dependent; (2) pH-dependent; (3) Na^+-, Cl^-- and energy-dependent; (4) inhibited by BT structural analogues; (5) sensitive to monocarboxylate transporter 1 (MCT1) inhibitors; and (6) insensitive to DIDS and amiloride. IEC-6 cells express both MCT1 and Na^+-coupled monocarboxylate transporter 1 (SMCT1) mRNA. We conclude that ^{14}C-BT uptake by IEC-6 cells mainly involves MCT1, with a small contribution of SMCT1. Acute exposure to ethanol, acetaldehyde, indomethacin, resveratrol and quercetin reduced ^{14}C-BT uptake. Chronic exposure to resveratrol and quercetin reduced ^{14}C-BT uptake but had no effect on either MCT1 or SMCT1 mRNA levels. Uptake of ^{14}C-BT by FHC cells was time- and concentration-dependent but pH-, Na^+-, Cl^-- and energy-independent and insensitive to BT structural analogues and MCT1 inhibitors. Although MCT1 (but not SMCT1) mRNA expression was found in FHC cells, the characteristics of ^{14}C-BT uptake by FHC cells did not support either MCT1 or SMCT1 involvement. In conclusion, uptake characteristics of ^{14}C-BT differ between IEC-6 and FHC cells. IEC-6 cells demonstrate MCT1- and SMCT1-mediated transport, while FHC cells do not.

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Keywords Butyrate uptake · Nontransformed intestinal epithelial cell · Monocarboxylate transporter type 1 · Xenobiotics

Introduction

The short-chain fatty acid butyrate (BT) is one of the main end products of anaerobic bacterial fermentation of dietary fiber within the human colon (Wong et al. 2006; Hamer et al. 2008). BT plays a key role in colonic epithelial homeostasis by having multiple regulatory roles at that level, including (1) being the main energy source for colonocytes, (2) inhibiting colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation), (3) promoting growth and proliferation of normal colonic epithelial cells, (4) stimulating fluid and electrolyte absorption, (5) inhibiting colon inflammation and oxidative stress and (6) improving the colonic defence barrier function (Wong et al. 2006; Hamer et al. 2008).

BT is transported into colonic epithelial cells by two specific carrier-mediated transport systems, an electroneutral H^+-coupled monocarboxylate cotransporter (MCT1, SLC16A1) (Halestrap and Meredith 2004; Morris and Felmlee 2008) and an Na^+-coupled monocarboxylate cotransporter (SMCT1, SLC5A8) (Gupta et al. 2006). As mentioned, one of the proposed beneficial effects of BT on human intestinal health is the prevention/inhibition of colon carcinogenesis (Park et al. 2005; Martínez et al. 2008). In agreement with this fact, both MCT1 (Cuff et al. 2005) and SMCT1 (Gupta et al. 2006) were recently proposed to function as tumor suppressors, the ability of these transporters to mediate the entry of BT into colonic epithelial

cells underlying their potential tumor-suppressor effect. Interestingly enough, the characteristics of BT colonic epithelial transport have been almost exclusively studied by using colon adenocarcinoma cell lines (Caco-2 or HT-29) (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). However, knowledge on the characteristics of BT uptake in noncarcinogenic cell lines seems important in the context of the distinct effect of BT in carcinogenic and noncarcinogenic cells. Indeed, although BT presents, in many tumor cells, an anticarcinogenic effect which involves induction of differentiation and apoptosis and inhibition of proliferation, its effect on noncarcinogenic cells is contrary, this phenomenon being referred as the "BT paradox" (Hamer et al. 2008).

Additionally, because BT plays an essential role in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important (Cuff and Shirazi-Beechey 2004). In this context, MCT1-mediated intestinal epithelial absorption of BT is known to be upregulated by its substrate, BT (Cuff et al. 2002); enhanced by leptin (Buyse et al. 2002), phorbol 12-myristate 13-acetate (Alrefai et al. 2004), protein kinase C (Saksena et al. 2009a), somatostatin (Saksena et al. 2009b), and caffeine and acetylsalicylic acid (Gonçalves et al. 2009); and inhibited by enteropathogenic *Escherichia coli* (Borthakur et al. 2006), interferon-γ and tumor necrosis factor-α (Thibault et al. 2007), theophylline, tetrahydrocannabinol, MDMA (ecstasy), acetaldehyde and indomethacin (Gonçalves et al. 2009), as well as by some polyphenolic compounds (Konishi et al. 2003; Vaidyanathan and Walle 2003; Shim et al. 2007; Gonçalves et al. in press). Information on the regulation of SMCT1 is even more limited. SMCT1 is known to be inhibited by some nonsteroidal anti-inflammatory drugs (NSAIDs) (Itagaki et al. 2006), by the absence of gut commensal bacteria (Cresci et al. 2010) and by tumor necrosis factor-α (Borthakur et al. 2010) and stimulated by some other NSAIDs (Ananth et al. 2010), by activin A (Zhang et al. 2010) and by the probiotic *Lactobacillus plantarum* (Borthakur et al. 2010). However, the above-mentioned studies concerning regulation of BT intestinal uptake were also done using colon adenocarcinoma cell lines only. Because BT exerts distinct effects in carcinogenic and noncarcinogenic cells (see above) and SMCT1 expression has been reported to be silenced in colorectal carcinoma and colon cancer cell lines (Ganapathy et al. 2008), it seemed interesting to compare also the effect of some of these compounds upon BT uptake in nontransformed intestinal epithelial cell lines.

Thus, the aim of this study was to characterize the uptake of BT by nontransformed intestinal epithelial cell lines and to test the effect of a series of drugs upon it. For this, we characterized ^{14}C-BT uptake by a rat small intestinal epithelial cell line (IEC-6) and by a fetal human colonic epithelial cell line (FHC) and tested the acute and chronic effect of drugs upon it. IEC-6 and FHC cells were chosen based on their nontransformed intestinal epithelial origin. Knowledge of the characteristics of BT uptake by these cell lines was very scarce, and nothing was known concerning MCT1 and SMCT1 expression (Lecona et al. 2008; Borthakur et al. 2010).

Materials and Methods

IEC-6 Cell Culture

The IEC-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-111; Braunschweig, Germany) and used between passages 19 and 34. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and cultured in Dulbecco's modified Eagle medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma, St. Louis, MO). Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, cells were removed enzymatically (0.05% trypsin-EDTA, 5 min, 37°C), split 1:3 and subcultured in plastic culture dishes (21 cm^2; ⌀ 60 mm; Corning Costar, Corning, NY). For uptake studies, IEC-6 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ⌀ 16 mm, Corning Costar), and the experiments were performed 9 days after the initial seeding (90–100% confluence). For 24 h before the experiments, the cell medium was made free of fetal calf serum and insulin.

FHC Cell Culture

The FHC cell line was obtained from the American Type Culture Collection (37-HTB; ATCC, Rockville, MD) and used between passages 21 and 26. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and cultured in DMEM:F12 Ham's nutrient mixture (1:1), supplemented with 10% fetal bovine serum, 10 mM HEPES, 1.2 g $NaHCO_3$, 10 ng/ml cholera toxin, 5 µg/ml insulin, 5 ng/ml transferrin, 100 ng/ml hydrocortisone, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days, and the culture was split every 15–16 days. For subculturing, cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split 1:2 and subcultured in plastic culture dishes (21 cm^2; ⌀ 60 mm, Corning Costar). For uptake studies, FHC cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ⌀ 16 mm, Corning Costar), and

the experiments were performed 15–20 days after the initial seeding (90–100% confluence). For 24 h before the experiments, the cell medium was made free of fetal calf serum.

Determination of ^{14}C-BT Uptake by IEC-6 and FHC Cells

Uptake experiments were performed with cells incubated in glucose-free Krebs (GFK) buffer containing (in mM) 125 NaCl, 25 $NaHCO_3$, 4.8 KCl, 0.4 K_2HPO_4, 1.6 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$ and 20 HEPES (pH 7.0, 7.5 or 8.0) or 20 MES (pH 5.5 and 6.5). In most of the experiments, a buffer with pH 6.5 was used. Initially, the culture medium was aspirated and the cells were washed with 0.3 ml buffer at 37°C. Then, cell monolayers were preincubated for 20 min in 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.3 ml medium at 37°C containing ^{14}C-BT (10 or 20 µM, except in kinetic experiments). Incubation was stopped after 3 min (except in time-course experiments) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold buffer. Cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

For characterization of ^{14}C-BT uptake by the cells, the effect of several drugs, medium pH or ionic composition was tested by preincubating and incubating cells with ^{14}C-BT in the presence of the compounds or conditions to be tested.

Acute and Chronic Effect of Drugs on ^{14}C-BT Uptake by IEC-6 Cells

The concentrations of compounds to test were chosen on the basis of previous works from our group (Araújo et al. 2008; Gonçalves et al. 2009, in press).

Acute Effect of Compounds

The acute effect of compounds on ^{14}C-BT uptake was tested by preincubating (20 min) and incubating (3 min) cells with ^{14}C-BT in the presence of the compounds to be tested.

Chronic Effect of Compounds

The chronic effect of compounds on ^{14}C-BT uptake was tested by cultivating cell cultures at 6–8 days of age (90–95% confluence) in culture medium in the presence of the compounds to be tested. The medium was renewed daily, and the transport experiments were performed after 48 h. Transport experiments were identical to the experiments described above, except that there was no preincubation period and cells were incubated with ^{14}C-BT in the absence of drugs.

Real-Time Quantitative Reverse-Transcription PCR

Total RNA was extracted from control FHC and IEC-6 cells and from chronically treated IEC-6 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 10 µg of resulting DNA-free RNA was reverse-transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen) in 40 µl of final reaction volume, according to the manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. For quantitative real-time PCR, 2 µl of the 40-µl reverse transcription reaction mixture was used. For the calibration curve, FHC and IEC-6 standard cDNA was diluted in five different concentrations.

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ). We set up 20-µl reactions in microcapillary tubes using 0.5 µM of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification (95°C for 10 s, annealing temperature [AT] for 15 s and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment) repeated 50 times, a melting curve program ([AT + 10]°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) and a cooling step to 40°C (30 s). ATs and primer sequences are indicated in Table 1. Data were analyzed using LightCycler® 4.05 analysis software (Roche, Mannheim, Germany).

Protein Determination

The protein content of cell monolayers was determined as described by Bradford (1976), using human serum albumin as standard.

Calculation and Statistics

For analysis of the time course of ^{14}C-BT uptake, the parameters of the equation $A(t) = k_{in}/k_{out}\left(1 - e^{-k_{out}\cdot t}\right)$ were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (Muzyka et al. 2005). A(t) represents the accumulation of

Table 1 Primer sequences and annealing temperatures (ATs) used for real-time qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
hGAPDH	Fwd ATG GAG AAG GCT GGG GCT CAT	65
	Rev GAC GAA CAT GGG GGC ATC AG	
hMCT1	Fwd CAC CGT ACA GCA ACT ATA CG	60
	Rev CAA TGG TCG CCT CTT GTA GA	
hSMCT1	Fwd CTC CCG GTG TTC TAC AAA CTG	65
	Rev GGG CAG GGG CAT AAA TAA C	
rGAPDH	Fwd GGC ATC GTG GAA GGG CTC ATG AC	72
	Rev ATG CCA GTG AGC TTC CCG TTC AGC	
rMCT1[a]	Fwd CAG TGC AAC GAC CAG TGA ATG TG	69
	Rev ATC AAG CCA CAG CCA GAC AGG	
rSMCT1	Fwd CGG GAT CAC CAG CAC CTA C	66
	Rev GCA GGG GCA TAA ATC ACA ATC	

hGAPDH human glyceraldehyde-3-phosphate dehydrogenase, *hMCT1* human monocarboxylate transporter type 1, *hSMCT1* human Na^+-coupled monocarboxylate transporter type 1, *rGAPDH* rat glyceraldehyde-3-phosphate dehydrogenase, *rMCT1* rat monocarboxylate transporter type 1, *rSMCT1* rat Na^+-coupled monocarboxylate transporter type 1, Fwd forward, Rev reverse

[a] Primer sequence obtained from Thibault et al. (2007)

^{14}C-BT at time t; k_{in} and k_{out} are the rate constants for inward and outward transport, respectively; and t is the incubation time. A_{max} corresponds to the accumulation (A[t]) at steady state ($t \rightarrow \infty$). k_{in} is given in picomoles per milligram of protein per minute (pmol/[mg protein·min]) and k_{out} in minutes (min^{-1}). In order to obtain clearance values, k_{in} was converted to microliters per milligram of protein per minute (µl/[mg protein·min]). For analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis-Menten equation were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (Muzyka et al. 2005).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. The statistical significance of the difference between two groups was evaluated by Student's *t*-test; statistical analysis of the difference between various groups was evaluated by ANOVA, followed by the Bonferroni test. Differences were considered to be significant when $P < 0.05$.

Materials

^{14}C-BT (*n*-butyric acid, sodium salt, [1-^{14}C]; specific activity 30–60 mCi/mmol) was from Biotrend Chemikalien (Köln, Germany); acetylsalicylic acid, acetic acid sodium salt, alpha-cyano-4-hydroxycinnamic acid (4-CHC), amiloride hydrochloride, choline chloride, chrysin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), dinitrophenol, ethanol, (–)epigallocatechin-3-gallate (EGCG), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 4-(hydroxymercuri)benzoic acid sodium salt (pCMB), indomethacin, luteolin, ketobutyric acid sodium salt hydrate, L-lactic acid sodium salt, lithium chloride, 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), myricetin, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), propionic acid sodium salt, pyruvic acid sodium salt, sodium fluoride, quercetin dihydrate, resveratrol, rutin and trypsin-EDTA solution were all from Sigma; dimethylsulfoxide (DMSO) and Triton X-100 were from Merck (Darmstadt, Germany); fetal calf serum was from Invitrogen; acetaldehyde was from May & Baker (Dagenham, UK); and caffeine was from BDH Laboratory Chemicals (Poole, UK).

Drugs to be tested were dissolved in water, ethanol, DMSO or methanol, the final concentration of these solvents being 1% in the buffer or 0.1% in the culture media for acute or chronic treatments, respectively. Controls for these drugs were run in the presence of the solvent.

Results

Time and pH Dependence of ^{14}C-BT Uptake by IEC-6 Cells

In the first series of experiments, we determined the time course of ^{14}C-BT uptake by IEC-6 cells. For this, cells were incubated with ^{14}C-BT (10 µM) for various periods of time. As shown in Fig. 1a, IEC-6 cells accumulated ^{14}C-BT in a time-dependent way and uptake was linear with time for up to 3 min of incubation. Thus, in subsequent experiments, cells were exposed to ^{14}C-BT (10 µM) for 3 min in order to measure initial rates of uptake. Next, the pH dependence of ^{14}C-BT uptake was evaluated. Uptake of

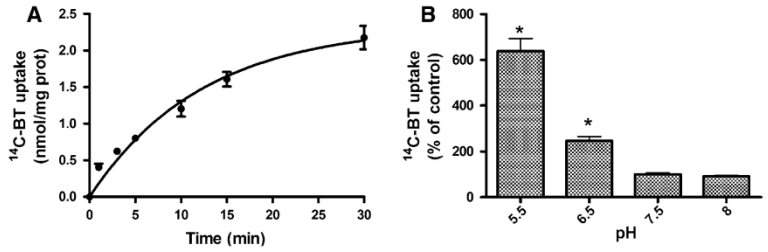

Fig. 1 Characteristics of ^{14}C-BT uptake by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT. **a** Time course of ^{14}C-BT uptake by IEC-6 cells incubated in GFK buffer (pH 6.5, $n = 5$–6). **b** pH dependence of ^{14}C-BT uptake (3 min). IEC-6 cells were preincubated and incubated in GFK buffer with pH ranging 5.5–8.0 ($n = 6$). Shown are arithmetic means ± SEM. *Significantly different from uptake at physiological pH (7.5)

^{14}C-BT (10 μM) was found to be highly pH-dependent, increasing as the pH decreased from 7.5 to 5.5 (Fig. 1b). Thus, in subsequent experiments, a pH of 6.5 was used.

Kinetics of ^{14}C-BT Uptake by IEC-6 Cells

The relationship between the initial rates of uptake of ^{14}C-BT and its concentration in the medium is represented in Fig. 2a. Rates were analyzed according to the Michaelis-Menten equation (see Materials and Methods). The evaluated kinetic parameters V_{max} and K_m were 69.5 ± 16.7 nmol/(mg prot · 3 min) and 4.0 ± 1.3 mM, respectively.

Pharmacological Characterization of ^{14}C-BT Uptake by IEC-6 Cells

To test for the dependence of ^{14}C-BT uptake on extracellular Na^+ and Cl^-, we measured uptake in the absence of NaCl, which was substituted by either LiCl, choline chloride or NaF. As shown in Fig. 2b, substitution of Cl^- with F^- caused a dramatic decrease in the uptake of ^{14}C-BT (≈60%). On the other hand, substitution of Na^+ with Li^+ caused a small decrease in the uptake of ^{14}C-BT (≈17%); but when Na^+ was substituted with choline, no change in the uptake of ^{14}C-BT was observed (Fig. 2b).

The effect of BT structural analogues (acetate and propionate, which, together with butyrate, constitute the major SCFA present in the lumen of the colon, and other monocarboxylates [L-lactate, pyruvate and α-ketobutyrate]) on the initial rates of ^{14}C-BT uptake was next determined. As shown in Fig. 2c, ^{14}C-BT uptake was significantly reduced by all of the monocarboxylates tested, with propionate showing the greatest inhibition (77%).

Moreover, we also tested the effect of a series of inhibitors. We verified that pCMB and NPPB, typical MCT1 inhibitors, caused a dramatic decrease in the uptake of ^{14}C-BT (≈80%). Moreover, the MCT1 inhibitor luteolin was also able to reduce ^{14}C-BT uptake. On the other hand, CHC caused a small (20%) but significant increase in the uptake of ^{14}C-BT. The classical anion exchange inhibitor DIDS and the typical inhibitor of apical Na^+/H^+ exchanger amiloride had no effect on ^{14}C-BT uptake. Finally, ^{14}C-BT uptake by IEC-6 cells was found to be highly energy-dependent as it was greatly reduced in the presence of dinitrophenol (Fig. 2d).

Modulation of ^{14}C-BT Uptake by IEC-6 Cells by Several Drugs

In this series of experiments, the acute and chronic effects of several therapeutic agents, abuse substances and polyphenolic compounds upon the uptake of ^{14}C-BT by IEC-6 cells were investigated. The compounds tested were previously found to affect uptake of ^{14}C-BT in human colon adenocarcinoma Caco-2 cells (Gonçalves et al. 2009, in press). The aim is to compare their effect on IEC-6 nontransformed cells with their effect on Caco-2 tumor cells.

Effect of Therapeutic and Abuse Compounds upon the Uptake of ^{14}C-BT by IEC-6 Cells

Acute Effect

As shown in Fig. 3a, caffeine (10 and 100 μM) and acetylsalicylic acid (1 and 5 mM) were devoid of effect upon ^{14}C-BT uptake. On the other hand, indomethacin (0.1 and 0.3 mM) concentration-dependently reduced ^{14}C-BT uptake, to a maximum of 44% of control. Moreover, ethanol and its metabolite acetaldehyde (10 and 100 mM) also reduced ^{14}C-BT uptake in a concentration-dependent manner, to a maximum of 74 and 23% of control, respectively.

Chronic Effect

These same compounds were tested over a 48-h period. Interestingly enough, none of these compounds (ethanol,

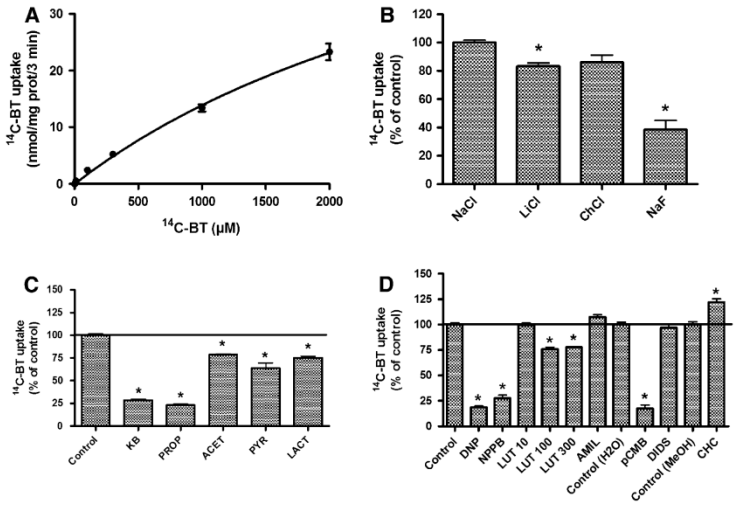

Fig. 2 Characteristics of ^{14}C-BT uptake by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT (except in the kinetic experiments) for 3 min in GFK buffer (pH 6.5). **a** Kinetics ($n = 7$). **b** Ionic dependence. NaCl in the preincubation and incubation GFK buffer (control) was isotonically replaced by either LiCl, choline chloride (ChCl) or NaF ($n = 9$–13). **c** Effect of BT structural analogues. Cells were incubated in the absence (control) or presence of 10 mM α-ketobutyrate (*KB*, $n = 6$), propionate (*PROP*, $n = 6$), acetate (*ACET*, $n = 6$), pyruvate (*PYR*, $n = 7$) and lactate (*LACT*, $n = 7$). **d** Effect of inhibitors. Cells were incubated in the absence (control) or presence of dinitrophenol 0.5 mM (*DNP*, $n = 7$); NPPB 0.5 mM ($n = 6$); luteolin 10, 100 or 300 μM (*LUT*, $n = 4$–8); amiloride 0.5 mM (*AMIL*, $n = 4$); pCMB 0.5 mM ($n = 6$); DIDS 0.5 mM ($n = 11$); or CHC 1 mM ($n = 8$). Shown are arithmetic means ± SEM. *Significantly different from control

acetaldehyde, caffeine, acetylsalicylic acid and indomethacin) caused a significant change in the uptake of ^{14}C-BT by IEC-6 cells (Fig. 3b).

Effect of Polyphenols upon the Uptake of ^{14}C-BT by IEC-6 Cells

Acute Effect

The acute effect of several different polyphenolic compounds was next investigated (Fig. 4a). Of these, resveratrol and quercetin (10–100 μM) were found to concentration-dependently reduce ^{14}C-BT uptake, to a maximum of 47 and 76% of control, respectively. In contrast, chrysin and myricetin did not affect uptake of ^{14}C-BT by IEC-6 cells.

Chronic Effect

As shown in Fig. 4b, EGCG, rutin and chrysin were devoid of effect on ^{14}C-BT uptake. However, quercetin (10 μM) and resveratrol (1 and 10 μM) reduced uptake of ^{14}C-BT by IEC-6 cells (to a maximum of 83 and 85% of control, respectively).

Characteristics of ^{14}C-BT Uptake by FHC Cells

The characteristics of ^{14}C-BT uptake were also investigated in human fetal colonic FHC cells. Uptake of ^{14}C-BT by FHC cells was time-dependent (Fig. 5a), and initial rates of uptake (which were measured by incubating cells with ^{14}C-BT for 3 min) displayed saturable kinetics, having a K_m of 1.22 ± 0.45 mM and a V_{max} of 108.9 ± 16.8 nmol/mg prot/3 min (Fig. 5b). Additionally, the initial rate of ^{14}C-BT uptake was found to be pH-independent, Na^+- and Cl^--independent, energy-independent and insensitive to both BT structural analogues (propionate, lactate and α-ketobutyrate) and MCT1 inhibitors (pCMB and NPPB) (Fig. 5c–e).

Real-Time qRT-PCR of MCT1 and SMCT1 mRNA in IEC-6 and FHC Cells

MCT1 and SMCT1 mRNA expression was investigated in both IEC-6 and FHC cells. IEC-6 cells express low levels of both transporters (Fig. 6a) and FHC cells express MCT1, but not SMCT1, mRNA (Fig. 6b).

Quantification of MCT1 and SMCT1 mRNA expression was also investigated in IEC-6 cells chronically treated with resveratrol (10 μM) or quercetin (10 μM). Treatment

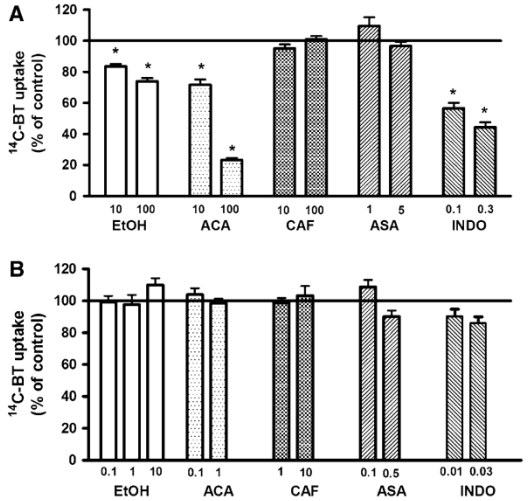

Fig. 3 Effect of several drugs upon the uptake of ^{14}C-BT by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min in GFK buffer (pH 6.5). **a** Acute effect. Cells were preincubated and incubated with ^{14}C-BT in the absence (control) or presence of ethanol (*EtOH*, 10–100 mM, $n = 9$), acetaldehyde (*ACA*, 10–100 mM, $n = 9$), caffeine (*CAF*, 10–100 μM, $n = 9$), acetylsalicylic acid (*ASA*, 1–5 mM, $n = 9$–12) or indomethacin (*IND*, 0.1–0.3 mM, $n = 9$). **b** Chronic effect. Cells were incubated with ^{14}C-BT after being cultivated for 48 h in the absence (control) or presence of EtOH (0.1–10 mM, $n = 10$–11), ACA (0.1–1 mM, $n = 11$–14), CAF (1–10 μM, $n = 9$), ASA (0.1–0.5 mM, $n = 15$) or IND (0.01–0.03 mM, $n = 15$). Shown are arithmetic means ± SEM. *Significantly different from control

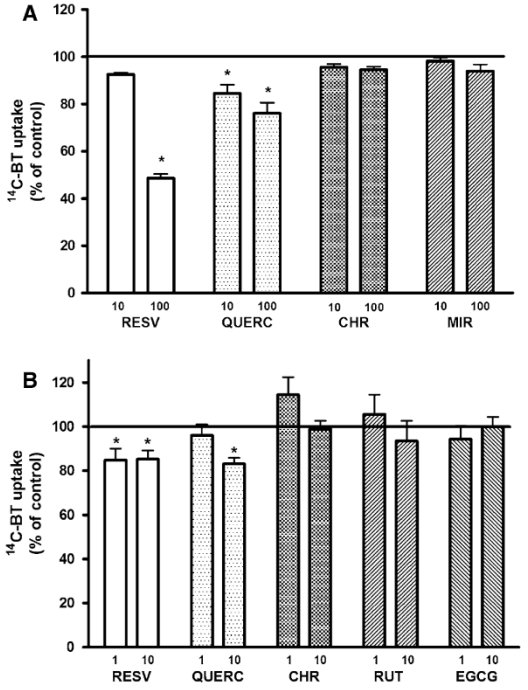

Fig. 4 Effect of several polyphenols upon uptake of ^{14}C-BT by IEC-6 cells incubated at 37°C with 10 μM ^{14}C-BT for 3 min in GFK buffer (pH 6.5). **a** Acute effect. Cells were preincubated and incubated with ^{14}C-BT in the absence (control) or presence of resveratrol (*RESV*, 10–100 μM, $n = 6$), quercetin (*QUERC*, 10–100 μM, $n = 6$), myricetin (*MYR*, 10–100 μM, $n = 9$) or chrysin (*CHR*, 10–100 μM, $n = 9$). **b** Chronic effect. Cells were incubated with ^{14}C-BT after being cultivated for 48 h in the absence (control) or presence of RESV (1–10 μM, $n = 9$), QUERC (1–10 μM, $n = 9$), rutin (*RUT*, 1–10 μM, $n = 8$), CHR (1–10 μM, $n = 8$–9) or EGCG (1–10 μM, $n = 8$–9). Shown are arithmetic means ± SEM. *Significantly different from control

with these compounds caused no change in expression levels of either MCT1 or SMCT1 (results not shown).

Discussion

The aim of this work was to characterize the uptake of BT in normal intestinal epithelial cells and to investigate its modulation by compounds previously found to affect BT uptake in human colon adenocarcinoma cells (Caco-2 cells).

Our first experiments were performed with the human fetal human colonic cell line FHC, which maintains the characteristics of normal human colonic cells (Siddiqui and Chopra 1984). This cell line was found to express MCT1 mRNA but not SMCT1 mRNA. However, characteristics of ^{14}C-BT uptake by FHC cells (namely, pH, Cl^- and energy independence and insensitivity to both BT structural analogues and MCT1 inhibitors) were quite distinct from those previously described in other intestinal epithelial cell lines, which are compatible with MCT1-mediated uptake (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). Thus, it is possible that although FHC cells express MCT1 mRNA, they do not express a functional protein (e.g., by lack of the chaperone CD147, which was recently found to be necessary for proper membrane expression and activity of MCT1 (Kirk et al. 2000; Su et al. 2009)) and that BT uptake in FHC cells involves a mechanism distinct from MCT1 and SMCT1 (e.g., a BT/HCO_3^- exchanger (Harig et al. 1996; Ritzhaupt et al. 1998; Schröder et al. 2000) or an organic anion transporter [OAT] (Anzai et al. 2006)). For this reason, FHC cells did not seem to be a good cell model to investigate BT uptake, and we decided to investigate ^{14}C-BT uptake in another nontransformed intestinal cell line.

The rat normal intestinal epithelial cell line IEC-6 was established from crypts of rat small intestinal cells by Quaroni et al. (1979). Although they were originally

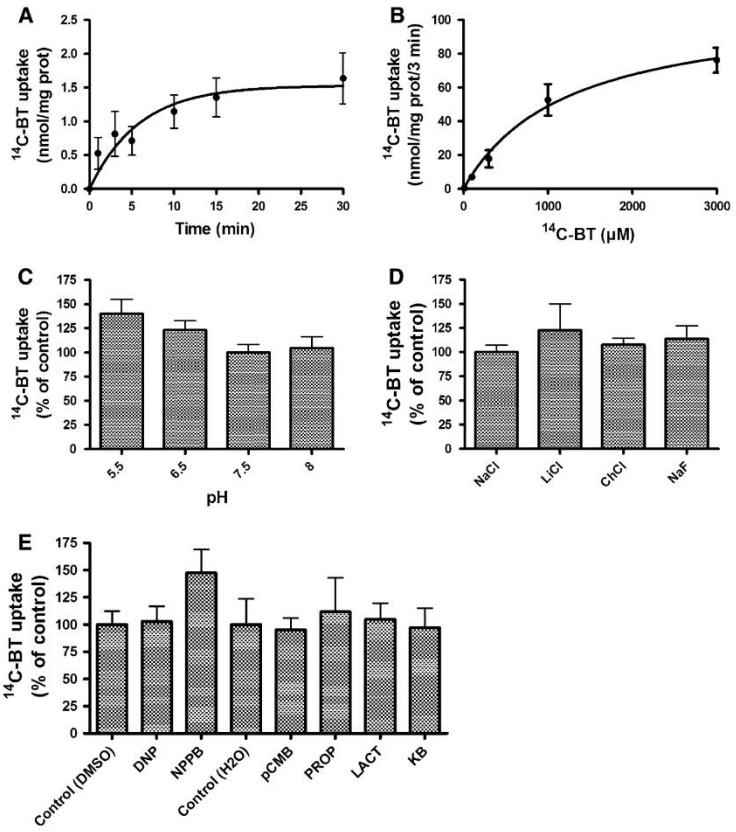

Fig. 5 Characteristics of ^{14}C-BT uptake by FHC cells incubated at 37°C in GFK buffer. **a** Time course of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT (pH 6.5, $n = 5$–6). **b** Kinetics of ^{14}C-BT uptake by cells incubated for 3 min with increasing concentrations of ^{14}C-BT (pH 6.5) ($n = 4$). **c** pH dependence of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT for 3 min. Extracellular pH in the preincubation and incubation GFK buffer ranged 5.5–8.0 ($n = 8$–9). **d** Ionic dependence of ^{14}C-BT uptake by cells incubated with 20 μM ^{14}C-BT for 3 min. NaCl in the preincubation and incubation GFK buffer (control. pH 6.5) was isotonically replaced by either LiCl, choline chloride (ChCl) or NaF ($n = 6$). **e** Effect of drugs on ^{14}C-BT uptake. Cells were incubated with 20 μM ^{14}C-BT for 3 min in the absence (control) or presence of dinitrophenol 10 mM (*DNP*, $n = 6$), 5-nitro-2-(3-phenylpropylamino)benzoate 0.5 mM (*NPPB*, $n = 5$), *p*-chloromercuribenzoate 0.5 mM (*pCMB*, $n = 6$), propionate 10 mM (*PROP*, $n = 5$), lactate 10 mM (*LACT*, $n = 6$) or α-ketobutyrate 10 mM (*KB*, $n = 6$). Shown are arithmetic means ± SEM

described as sharing many undifferentiated characteristics of immature intestinal cells, when IEC-6 cells are grown in postconfluent culture, they develop structural changes and differentiation from a crypt cell-like to an enterocyte-like phenotype (Wood et al. 2003). IEC-6 cells have been used in numerous studies, including studies on the intestinal absorption of nutrients (e.g., Inui et al. 1980; Jakobs and Paterson 1986; Said et al. 1997; Fujita et al. 2000; Murota et al. 2001; Fraga et al. 2002).

Uptake of ^{14}C-BT by IEC-6 cells was found to be (1) time- and concentration-dependent; (2) pH-dependent, with uptake increasing with decreasing pH; (3) Na^+- and Cl^--dependent; (4) energy-dependent; (5) inhibited by several BT structural analogues (propionate, lactate, acetate, pyruvate and α-ketobutyrate); (6) inhibited by the MCT inhibitors pCMB, NPPB and luteolin and enhanced by CHC; and (7) insensitive to a classic anion exchange inhibitor (DIDS) and to a typical inhibitor of apical Na^+/H^+ exchanger (amiloride).

As mentioned before, the characteristics of BT colonic epithelial transport have been almost exclusively studied by using colon adenocarcinoma cell lines (Caco-2 or HT-29 cells) (Hadjiagapiou et al. 2000; Stein et al. 2000; Lecona et al. 2008; Gonçalves et al. 2009). Interestingly

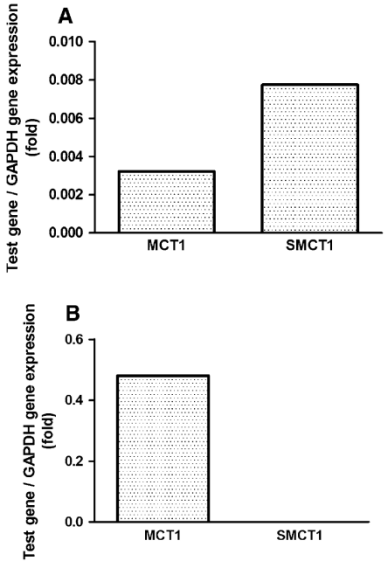

Fig. 6 Gene-expression levels of MCT1 and SMCT1 in IEC-6 cells (**a**) and FHC cells (**b**). MCT1 and SMCT1 levels were quantified by real-time qRT-PCR, as described in Materials and Methods. Results are shown as arithmetic means corresponding to the expression of MCT1 or SMCT1 relative to GAPDH ($n = 2$)

enough, the characteristics of ^{14}C-BT uptake in Caco-2 (recently described by our group (Gonçalves et al. 2009)) and IEC-6 cells show a high degree of similarity—namely, the time, pH and Cl^- dependence, the saturation kinetics with very similar K_m (4.0 and 2.8 mM for IEC-6 and Caco-2 cells, respectively), the inhibition by BT structural analogues and by the MCT1 inhibitors pCMB and NPPB and the lack of sensitivity to DIDS and CHC. The only difference between uptake of this compound by the two cell lines relates to its dependence on extracellular Na^+: Whereas uptake by Caco-2 cells is Na^+-independent, uptake by IEC-6 cells is slightly (17%) dependent on Na^+. Interestingly enough, we verified that IEC-6 cells express both MCT1 and SMCT1 mRNA. Thus, we conclude that uptake of ^{14}C-BT by IEC-6 cells seems to be mainly mediated by MCT1, with a small contribution of SMCT1.

It has been reported that SMCT1 is able to transport monocarboxylates in an Na^+-coupled manner when expressed in *Xenopus laevis* oocyes (Coady et al. 2004). However, most of the previous studies could not show Na^+-coupled BT transport in colonic cells (Hadjiagapiou et al. 2000; Stein et al. 2000; Gonçalves et al. 2009), with the exception of the work of Lecona et al. (2008), showing an Na^+-dependent high-affinity component of BT uptake in the human colon BCS-TC2 adenocarcinoma cell line. Interestingly enough, SMCT1 mRNA and Na^+-dependent BT uptake were very recently described in IEC-6 cells (Borthakur et al. 2010). Thus, our work fully confirms these findings. Nevertheless, we also think that the involvement of SMCT1 in BT transport in the normal colon is unlikely, given the low K_m (in the micromolar range) of SMCT1 for BT. Indeed, BT luminal concentrations in the normal colon are much higher (in the millimolar range), suggesting that SMCT1 could have a much less important role in BT transport than MCT1 (Thangaraju et al. 2008; Thibault et al. 2010).

Because BT plays an essential role in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important. In the past few years, important data concerning this subject have been obtained (see Introduction). However, these reports have employed colon adenocarcinoma cells only. Thus, it seemed important to investigate regulation of BT uptake also in normal intestinal epithelial cells, so the effect of a series of compounds recently found to affect BT uptake in Caco-2 cells (Gonçalves et al. 2009, in press) was investigated in the second part of this study.

Interestingly enough, we verified that several of the tested compounds affected ^{14}C-BT uptake by IEC-6 cells. Namely, acute exposure of IEC-6 cells to ethanol, acetaldehyde, indomethacin, resveratrol and quercetin and chronic exposure to resveratrol and quercetin reduced ^{14}C-BT uptake.

Epidemiological data have identified chronic alcohol consumption as a significant risk factor for colorectal cancer. Although ethanol is not carcinogenic in animal models, its bacterial fermentation in the colon produces acetaldehyde, which is highly toxic, mutagenic and carcinogenic (Pöschl and Seitz 2004; Bongaerts et al. 2006; Seitz and Homann 2007). In IEC-6 cells, acute exposure to ethanol and to its metabolite acetaldehyde reduced ^{14}C-BT uptake in a concentration-dependent manner. From these results, we can speculate that interference with BT uptake by the colonic epithelium might contribute to the colorectal cancer promoter effect of ethanol and acetaldehyde. Previously, acute exposure to acetaldehyde was also found to reduce ^{14}C-BT uptake in Caco-2 cells in a concentration-dependent and competitive manner (Gonçalves et al. 2009).

The NSAIDs emerged as a new perspective in tumor therapy as well as in cancer prevention (Tuynman et al. 2004; Elwood et al. 2009). Some NSAIDs are transportable substrates for MCTs but nontransportable blockers of SMCT1 (Coady et al. 2004; Choi et al. 2005; Itagaki et al. 2006). In Caco-2 cells, acute exposure to the NSAIDs acetylsalicylic acid and indomethacin concentration-dependently inhibited ^{14}C-BT uptake, and it was concluded that these two compounds are inhibitors of MCT1-mediated transport of BT (Gonçalves et al. 2009). Indomethacin also inhibited the uptake of ^{14}C-BT in IEC-6 cells (and

more potently that in Caco-2 cells). However, acetylsalicylic acid had no effect. Although we have at the present moment no explanation for this difference, it may be related to a differential regulation of MCT1 in these two cell lines (see below).

For ethanol, acetaldehyde and indomethacin, the observation of BT uptake inhibition after acute exposure but not after chronic exposure suggests that changes in the intrinsic activity of either MCT1 or SMCT1 induced acutely by these agents disappear after chronic exposure.

The flavonoid quercetin and the stilbene resveratrol were previously shown to be MCT1 inhibitors in Caco-2 cells (Shim et al. 2007; Gonçalves et al. in press), although their inhibitory effect disappeared after chronic exposure (Gonçalves et al. in press). In IEC-6 cells, these compounds were found to inhibit the uptake of ^{14}C-BT both acutely and chronically, thus demonstrating a more consistent inhibitory effect upon BT uptake in noncarcinogenic cells compared with carcinogenic ones. The lack of effect of chronic resveratrol and quercetin upon MCT1 and SMCT1 mRNA levels suggests that their effect does not result from changes in MCT1 or SMCT1 transcription rates but, rather, from changes in either functional protein levels or protein intrinsic activity.

Further comparison of the effect of the tested compounds on IEC-6 and Caco-2 cells gives interesting results. For some of the compounds, a similar effect in both cell lines was observed (acute acetaldehyde, indomethacin, resveratrol and quercetin). Moreover, and similar to what was verified with Caco-2 cells (Gonçalves et al. 2009), some compounds (ethanol, acetaldehyde and indomethacin) affected BT uptake when tested acutely but lost their effect after chronic exposure. However, for most of the compounds, the effect was found to be dramatically different in these two cell lines (acute ethanol, caffeine, acetylsalicylic acid, chrysin and myricetin and chronic caffeine, resveratrol, quercetin, chrysin, rutin and EGCG). Thus, ^{14}C-BT uptake in IEC-6 and Caco-2 cells seems to be differentially modulated by several distinct xenobiotics. These results are very interesting in the context of colon carcinogenesis as the effect of BT in many tumor cells (an anticarcinogenic effect which involves induction of differentiation and apoptosis and inhibition of proliferation) and noncarcinogenic cells is opposite, this phenomenon being referred as the "BT paradox" (Hamer et al. 2008). Thus, a comparison between the effect of a given compound upon BT uptake in carcinogenic and noncarcinogenic cell lines seems important. On the one hand, inhibition of BT uptake in tumor and nontumor colon cells will most probably have opposite effects in the context of carcinogenesis/anticarcinogenesis. On the other hand, a dramatic difference in the effect of a given compound on tumor and nontumor cells might be of interest. In this context, the effect of chronic caffeine, quercetin and EGCG (increasing BT uptake in Caco-2 while having no effect or decreasing uptake in IEC-6 cells) appears very interesting.

Finally, as uptake of BT by IEC-6 cells (present study) and Caco-2 cells (Gonçalves et al. 2009) seems to be mainly mediated by the same carrier-mediated mechanism, MCT1, the distinct effect of most of the xenobiotics tested upon ^{14}C-BT uptake in these two cell lines points to the possibility that MCT1 is differentially regulated in IEC-6 and Caco-2 cells. Knowing that these xenobiotics interfere with several distinct intracellular regulatory pathways, we hypothesize that this differential regulation might occur at several distinct levels (e.g., at the level of MCT1 gene transcription, protein synthesis or phosphorylation/dephosphorylation).

In conclusion, uptake of ^{14}C-BT by FHC and IEC-6 cells shows rather distinct characteristics. Uptake by IEC-6 cells seems to involve mainly MCT1, with a small contribution from SMCT1. Therefore, the IEC-6 cell line may be used to study regulation of SMCT1 expression and function. On the contrary, uptake by FHC cells seems to involve neither MCT1 nor SMCT1, and this cell line does not seem useful for examining BT intestinal transport. Moreover, uptake of ^{14}C-BT by IEC-6 cells is inhibited by either acute or chronic exposure to a series of xenobiotics (ethanol, acetaldehyde, indomethacin, resveratrol and quercetin). Finally, the distinct effect of some of the xenobiotics tested (chronic caffeine, quercetin and EGCG) upon ^{14}C-BT uptake by IEC-6 (no effect or decrease) and tumoral (Caco-2) cells (increase) (Gonçalves et al. 2009) might be of interest in the context of colon carcinogenesis.

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III - The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein

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The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein

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Gonçalves P, Gregório I, Martel F. The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein. *Am J Physiol Cell Physiol* 301: C984–C994, 2011. First published July 20, 2011; doi:10.1152/ajpcell.00146.2011.—Colorectal cancer is one of the most common cancers worldwide. Butyrate (BT) plays a key role in colonic epithelium homeostasis. The aim of this work was to investigate the possibility of BT being transported by P-glycoprotein (MDR1), multidrug resistance proteins (MRPs), or breast cancer resistance protein (BCRP). Uptake and efflux of ^{14}C-BT and ^{3}H-folic acid were measured in Caco-2, IEC-6, and MDA-MB-231 cell lines. mRNA expression of BCRP was detected by RT-PCR. Cell viability, proliferation, and differentiation were quantified with the lactate dehydrogenase, sulforhodamine B, and alkaline phosphatase activity assays, respectively. In both IEC-6 cells and Caco-2 cells, no evidence was found for the involvement of either MDR1 or MRPs in ^{14}C-BT efflux from the cells. In contrast, several lines of evidence support the conclusion that BT is a substrate of both rat and human BCRP. Indeed, BCRP inhibitors reduced ^{14}C-BT efflux in IEC-6 cells, both BT and BCRP inhibitors significantly decreased the efflux of the known BCRP substrate ^{3}H-folic acid in IEC-6 cells, and BCRP inhibitors reduced ^{14}C-BT efflux in the BCRP-expressing MDA-MB-231 cell line. In IEC-6 cells, combination of BT with a BCRP inhibitor significantly potentiated the effect of BT on cell proliferation. The results of this study, showing for the first time that BT is a BCRP substrate, are very important in the context of the high levels of BCRP expression in the human colon and the anticarcinogenic and anti-inflammatory role of BT at that level. So, interaction of BT with BCRP and with other BCRP substrates/inhibitors is clearly of major importance.

butyrate efflux; colorectal cancer; IEC-6 cells; anticarcinogenic effect

COLORECTAL CANCER (CRC) is a leading cause of cancer death in occidental countries (36). Butyrate (BT), a product of intestinal flora fermentation of dietary fiber, has a protective role in the prevention and progression of colorectal carcinogenesis (57). Indeed, this short-chain fatty acid plays a key role in colonic epithelium homeostasis, by having multiple important roles at that level: *1*) it is the main energy source for colonocytes, *2*) it promotes growth and proliferation of normal colonic epithelial cells, *3*) it inhibits colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation), *4*) it inhibits colon inflammation and oxidative stress, *5*) it improves the colonic defence barrier function, and *6*) it stimulates fluid and electrolyte absorption (reviews in Refs. 31, 68).[1]

The mechanism by which BT inhibits colon carcinogenesis seems to involve various effects on gene expression, which are mainly attributed to its capacity to act as a histone deacetylase inhibitor (HDAC), leading to hyperacetylation of histones and to increased accessibility of transcription factors to DNA promoters (11). Moreover, BT also influences posttranslational modifications, including DNA methylation (12), histone methylation (51), and hyperacetylation of nonhistone proteins (67).

Because BT plays a central role in colonic cellular metabolism and maintenance of tissue homeostasis, and because many cellular effects of BT are dependent on its intracellular concentration (e.g., inhibition of histone deacetylases; please see above), knowledge of the mechanisms involved in its membrane transport seem particularly important.

BT is known to be transported into colonic epithelial cells by two specific carrier-mediated transport systems, the electroneutral H^+-coupled monocarboxylate transporter 1 (MCT1) (45) and the Na^+-coupled monocarboxylate cotransporter (SMCT1) (26). MCT1 (7) and SMCT1 (27) were recently proposed to function as tumor suppressors, the ability of these transporters to mediate the entry of BT into colonic epithelial cells underlying their potential tumor suppressor effect. However, BT cellular pools are not only dependent on the above BT uptake systems but also depend on efflux transporters, which are able to remove BT from the cells.

The ATP-binding cassette (ABC) transporter superfamily contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, sterols, and drugs (16, 50). Overexpression of certain ABC transporters [e.g., P-glycoprotein (MDR1; encoded by ABCB1), multidrug resistance protein 1 (MRP1; encoded by ABCC1), and the breast cancer resistance protein (BCRP; encoded by ABCG2)] occur in cancer cell lines and tumors that are multidrug resistant (reviews in Refs. 14, 56). In addition to their role in multidrug resistance, ABC transporters such as MDR1, MRPs, and BCRP are also expressed in non-malignant tissues. The human intestinal tract expresses high levels of MDR1, MRPs, and BCRP (e.g., Refs. 18, 59, 61), and these efflux transporters are believed to be involved in limiting drug absorption, bioavailability, and toxicity (e.g., Refs. 16, 50). Interestingly enough, BT is known to induce the expression of MDR1 (17, 43, 44). Moreover, other histone deacetylase inhibitors such as depsipeptide (71), vorinostat, valproic acid (19), and trichostatin A (37) also induce MDR1-dependent resistance in human cancer cells, and depsipeptide and trichostatin A were described as MDR1 substrates (54, 73).

So, because nothing is known concerning the putative interaction of BT with ABC transporters, the aim of this work was to investigate the possibility of BT being transported by MDR1, MRPs, or BCRP. We demonstrate that BT is a BCRP substrate and that inhibition of BCRP significantly potentiates the effect of BT on cell proliferation. Given the anticarcino-

[1] This article is the topic of an Editorial Focus by Ravinder K. Gill and Pradeep K. Dudeja (22a).

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genic and anti-inflammatory role of BT at the intestinal epithelium and the high levels of expression of BCRP at that level, the interaction of BT with BCRP and other BCRP substrates/inhibitors is indeed of major importance.

MATERIALS AND METHODS

Caco-2 Cell Culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between *passage numbers 53–71*. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21-cm^2; diameter 60 mm; Corning Costar, Corning, NY). For use in experiments, Caco-2 cells were seeded (seeding density of 0.65×10^5 cells/cm^2) on 24-well plastic cell culture clusters (2-cm^2; diameter 16 mm; TPP, Trasadingen, Switzerland), and most experiments were performed 7 days after the initial seeding (90–100% confluence). In some experiments, 21-day-old Caco-2 cell cultures were used.

IEC-6 Cell Culture

The IEC-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-111, Braunschweig, Germany) and was used between *passage numbers 27–45*. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were cultured in Dulbecco's Modified Eagle's Medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; diameter 60 mm; Corning Costar). For use in experiments, IEC-6 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP), and experiments were performed 8–9 days after the initial seeding (90–100% confluence).

MDA-MB-231 Cell Culture

The MDA-MB-231 cell line [tumorigenic, highly metastatic breast cancer-derived cells; ERα(−)] was obtained from the American Type Culture Collection (HTB-26; ATCC, Rockville, MD) and used at *passage 29*. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; diameter 60 mm; Corning Costar). For use in experiments, MDA-MB-231 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; diameter 16 mm; TPP), and experiments were performed 7–8 days after the initial seeding (90–100% confluence).

Transport Studies

Transport experiments were performed with Caco-2 cells, IEC-6, and MDA-MB-231 incubated in glucose-free Krebs (GFK) buffer containing (in mM) 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4, and 20 MES, pH 6.5 (^{14}C-BT experiments) or pH 5.5 (^{3}H-FA experiments).

Influx transport experiments. Initially, the culture medium was aspirated, and the cells were washed twice with 0.3 ml of GFK buffer at 37°C. Then the cell monolayers were preincubated for 20 min with 0.3 ml of GFK buffer at 37°C, and uptake was initiated by the addition of 0.3 ml of GFK buffer at 37°C containing 10 μM ^{14}C-BT or 10 nM ^{3}H-FA. Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.5 ml of ice-cold GFK buffer. The cells were then solubilized with 0.3 ml of 0.1% (vol/vol) Triton X-100 (in 5 mM Tris·HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting. Drugs to be tested were present during both the preincubation and incubation periods.

Efflux transport experiments. Initially, the culture medium was aspirated, and the cells were washed twice with 0.3 ml of GFK buffer at 37°C. Then the cell monolayers were preincubated for 20 min with 0.3 ml of GFK buffer at 37°C, and uptake was initiated by the addition of 0.3 ml o GFK buffer at 37°C containing 10 μM ^{14}C-BT or 10 nM ^{3}H-FA. Incubation was stopped after 30 min by removing the incubation medium and rinsing the cells with 0.5 ml of ice-cold buffer. Then efflux was measured by incubating the cells with 0.3 ml of buffer at 37°C for 5 or 20 min. At the end of this period, the medium was collected, and the cells were solubilized with 0.3 ml of 0.1% (vol/vol) Triton X-100 (in 5 mM Tris·HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in both the efflux buffer and the cells was measured by liquid scintillation counting. Drugs to be tested were present during the efflux period only.

Chronic Treatment of the Cells

In some experiments, Caco-2 or IEC-6 cells were treated for 48 h with BT (2 or 5 mM in most of the experiments) or Ko143 (100 nM) before transport, proliferation, viability, and differentiation studies. These concentrations of BT chosen are well within the physiological range of concentrations of this compound in the human colon, since the concentration of short-chain fatty acids in the human colon may reach 70–130 mM after digestion of dietary fiber, with 20–30% of these corresponding to BT (9, 47), and BT concentrations in human feces were found to range from 11 to 25 mM (30, 66).

Protein Determination

The protein content of cell monolayers was determined as described (3), using human serum albumin as standard.

Quantification of Cellular Viability (Lactate Dehydrogenase Assay)

After the treatment period (48 h), cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular (culture) medium was measured spectrophotometrically by quantification of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described in Ref. 2.

Determination of Cellular Proliferation (Sulforhodamine B Assay)

After the treatment period (48 h), 62.5 μl of ice-cold 50% (wt/vol) trichloroacetic acid (TCA) were added to the culture medium (500 μl) on each well to fix cells (1 h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% (vol/vol) acetic acid. SRB was removed, and cultures were rinsed four times with 1% (vol/vol) acetic acid to remove residual dye. Plates were again air-dried, and the bound dye was then solubilized with 375 μl of 10 mM Tris·NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted to obtain absorbance values lower than 0.7.

Determination of Cellular Differentiation (Alkaline Phosphatase Activity Assay)

After the treatment period (48 h), cell differentiation was measured by quantification of alkaline phosphatase (ALP) activity, as previously described (48). ALP activity was determined spectrophotometrically by using *p*-nitrophenylphosphate as substrate, and the results were expressed as nmol *p*-nitrophenol·min^{-1}·mg protein^{-1}.

RT-PCR

Total RNA was extracted from IEC-6 cells using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics). Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen), according to manufacturer's instructions, and 10 μg of resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II (RT) and random hexamer primers (Invitrogen) in 40 μl of final reaction volume, according to the manufacturer's instructions. For paired negative controls, RT was omitted. Resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. Using 4 μl of this preparation, PCR was performed. The PCR mixture (50 μl) contained 0.5 μM per primer, 0.2 mM per dNTP, 2.3 mM $MgCl_2$, and 2 U of DyNAzyme II (Finnzymes, Keilaranta, Espoo, Finland). The primer pairs used for amplification and the predicted size of PCR products were as follows: 5′-CCA TCA CCA TCT TCC AGG AG-3′ (forward) and 5′-CCT GCT TCA CCA CCT TCTTG-3′ (reverse) for rat GAPDH (rGAPDH; 576 bp), and 5′-AGA GGG AGA TGT GCT AAG TTT-3′ (forward) and 5′-TGG TGA ATG GAG AAG ATG A-3′ (reverse) for rat BCRP (rBCRP; 633 bp). The thermocycling conditions for rGAPDH and rBCRP were 94°C for 2 min (1 cycle), 94°C for 20 s, 63°C for 60 s, 72°C for 40 s (30 cycles for rGAPDH and 35 cycles for rBCRP), and 72°C for 10 min (1 cycle). Individual PCR reaction products were run on 2.5% agarose gel and visualized with an ultraviolet transilluminator (UVP, Cambridge, UK) using ethidium bromide staining. PCR reaction products were recorded in a GelDOC-It Imaging System camera with the appropriate filters for ultraviolet light.

Calculation and Statistics

Arithmetic means are given with SE, and geometric means are given with 95% confidence limits. Statistical significance of the difference between two groups was evaluated by the Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the ANOVA test, followed by the Bonferroni test. Differences were considered to be significant when $P < 0.05$.

Materials

^{14}C-BT ([1-^{14}C]-*n*-butyric acid, sodium salt; specific activity 30–60 mCi/mmol), ^{3}H-folic acid ([3′,5′,7,9-^{3}H]folic acid potassium salt; specific activity 30 Ci/mmol) (Biotrend Chemikalien, Köln, Germany); antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), DMSO (dimethylsulfoxide), ethanol, fumitremorgin C (from *Neosartorya fischeri*), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), indomethacin, Ko143 [(3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl) 1,4-dioxopyrazino [1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester], MES (2-[*N*-morpholino]ethanesulfonic acid hydrate), nicotinamide adenine dinucleotide (NADH), p-nitrophenylphosphate, probenecid, quinidine sulfate, serum albumin, sodium butyrate, sodium pyruvate, sulforhodamine B, trichloroacetic acid sodium salt, trypsin-EDTA solution, verapamil hydrochloride, vinblastine sulfate (Sigma); fetal calf serum (Invitrogen); dimethylsulfoxide (DMSO), triton X-100 (Merck, Darmstadt, Germany).

Drugs to be tested were dissolved in water, DMSO, or ethanol; the final concentration of these solvents in the culture medium and GFK buffer being 1% and 0.1%, respectively. Controls for these drugs were run in the presence of the respective solvent.

RESULTS

To investigate the putative involvement of members of the ABC family of transporters in the handling of BT, we tested the effect of known inhibitors of some of these transporters on both ^{14}C-BT influx into and efflux out of Caco-2 and IEC-6 cells. The tested inhibitors were verapamil, quinidine and vinblastine (MDR1 inhibitors) (20), probenecid and indomethacin (MRPs inhibitors) (58, 60), and fumitremorgin C (FTC) and Ko143 (BCRP inhibitors) (1, 53).

We have previously shown that the apical uptake of ^{14}C-BT in both Caco-2 cells (25) and IEC-6 cells (23) was linear with time for up to 3 min of incubation. So, in the present work, cells were incubated with ^{14}C-BT for 3 min to measure initial rates of uptake.

Effect of Inhibitors of ABC Transporters On Influx and Efflux of ^{14}C-BT in 7-Day-Old Caco-2 Cells

The Caco-2 cell line expresses several efflux transporters such as MDR1, MDR3, MRP1–6, and BCRP (18, 59, 61, 70). So, in a first series of experiments, we determined the influence of inhibitors of different ABC transporters on initial rates of ^{14}C-BT uptake by 7-day-old Caco-2 cells. ^{14}C-BT uptake by Caco-2 cells was not affected by verapamil, vinblastine, indomethacin, FTC, and Ko143 (results not shown). However, quinidine (100 μM) and probenecid (500 μM) decreased ^{14}C-BT uptake (by 12 ± 2% and 15 ± 2%, respectively; $n = 8$).

Next, we examined the influence of the same compounds on the efflux of ^{14}C-BT from 7-day-old Caco-2 cells. None of the compounds tested had any significant effect on either the 5- or the 20-min efflux of ^{14}C-BT, with the exception of indomethacin (100 μM), which decreased the 5-min efflux by 17 ± 4% ($n = 11$–16).

Taken together, the results suggest that, in Caco-2 cells, there is no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in ^{14}C-BT efflux. MDR1 involvement was excluded because verapamil and vinblastine were not able to affect either ^{14}C-BT uptake or efflux, and BCRP involvement was excluded because its inhibitors (FTC and Ko143) were also devoid of effect on both the influx and efflux of ^{14}C-BT. Finally, MRPs involvement was also excluded because, although indomethacin decreased ^{14}C-BT efflux, it did not increase ^{14}C-BT influx, as expected if an exporter transporter is inhibited. Moreover, the fact that probenecid decreased ^{14}C-BT uptake suggests that probenecid and indomethacin are inhibiting a bidirectional transporter, which most probably corresponds to MCT1, SMCT1, or a member of the organic anion transporter family (8, 23, 25).

Effect of Inhibitors of ABC Transporters On Influx and Efflux of ^{14}C-BT in 21-Day-Old Caco-2 Cells

As shown in the previous section, inhibitors of distinct ABC transporters showed little effect on 7-day-old Caco-2 cells. Mariadason et al. (42) suggested a correlation between the degree of Caco-2 cell differentiation (which increases spontaneously with time in culture) and the resistance to BT

cellular effects. Mature colon epithelial cells have high levels of expression of MDR1 relative to most other cell types (37), and it is intriguing to speculate that this high intrinsic expression could lead to increased resistance to BT. So we decided to evaluate the effect of the same inhibitors of efflux transporters on the influx and efflux of ^{14}C-BT in well differentiated Caco-2 cells (21 days in culture).

Again, none of the compounds tested had any significant effect on uptake of ^{14}C-BT (results not shown). In relation to ^{14}C-BT efflux, the only significant effects were a slight increase in the presence of indomethacin (100 μM) and probenecid (500 μM) (9 ± 2% and 9 ± 3%, respectively; n = 12–16). These results suggest that, in well differentiated Caco-2 cells, there is also no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in ^{14}C-BT efflux.

Effect of Inhibitors of ABC Transporters On Influx and Efflux of ^{14}C-BT in IEC-6 Cells

The effects of BT on noncarcinogenic cells have been reported as contrary to the effects observed in tumor cell lines as to proliferation, differentiation, and apoptosis, this having been referred to as the "butyrate paradox" (31, 68). So, we next decided to evaluate the effect of ABC transport inhibitors on the influx and efflux of ^{14}C-BT in a noncarcinogenic cell line, the IEC-6 cells. These cells express MDR1, MRP1, MRP3, MRP4, and MRP5, and show MDR1- and MRPs-mediated transport (40, 64). Uptake of ^{14}C-BT by IEC-6 cells was not affected by verapamil or vinblastine. On the other hand, quinidine (100 μM), indomethacin (100 μM), and probenecid (500 μM) decreased ^{14}C-BT uptake (by 15–30%), and the BCRP inhibitors FTC (5 μM) and Ko143 (1 μM) increased ^{14}C-BT uptake (by 12–13%; Fig. 1*A*).

Next, we examined the influence of these same compounds on the efflux of ^{14}C-BT. As shown in Fig. 1*B*, quinidine (100 μM), vinblastine (100 μM), probenecid (500 μM), FTC (5 μM), and Ko143 (1 μM) were able to significantly decrease efflux of ^{14}C-BT (by 8–16%). The results obtained suggest that, in IEC-6 cells, there is no involvement of MDR1 and MRPs in ^{14}C-BT transport. Namely, MDR1 involvement is excluded because, although both quinidine and vinblastine inhibited the efflux of ^{14}C-BT, neither MDR1 inhibitor tested (verapamil, quinidine, and vinblastine) increased ^{14}C-BT uptake. The same was observed with inhibitors of MRPs. On the contrary, the involvement of BCRP in ^{14}C-BT efflux is strongly supported by the finding that both FTC and Ko143 increased uptake and decreased efflux of ^{14}C-BT, thus increasing the intracellular content of BT.

mRNA Expression of BCRP in IEC-6 Cells

Because BT was hypothesized to be a BCRP substrate in IEC-6 cells, we decided to confirm the mRNA expression of BCRP in these cells, which was not yet described. As expected, we demonstrated that IEC-6 cells express rBCRP mRNA (results not shown).

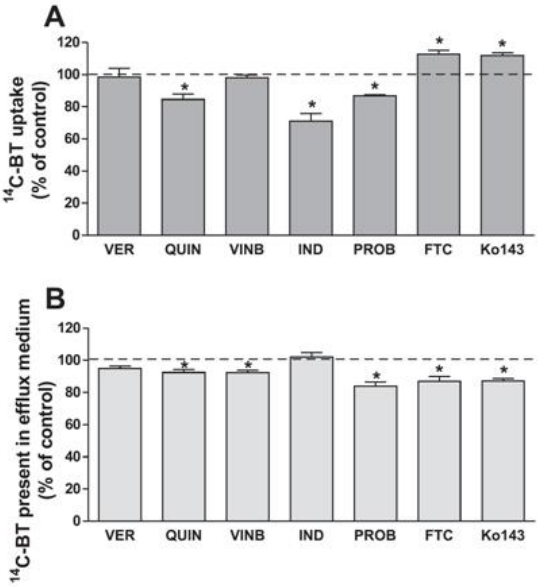


Fig. 1. Effect of various compounds on the apical uptake (*A*) and efflux (*B*) of ^{14}C-butyrate (BT) by IEC-6 cells. *A*: the cell monolayers were incubated at 37°C for 3 min with ^{14}C-BT (10 μM) in the absence (control) or presence of 20 μM verapamil (VER; n = 8), 100 μM quinidine (QUIN; n = 8), 100 μM vinblastine (VINB; n = 8), 100 μM indomethacin (IND; n = 8), 500 μM probenecid (PROB; n = 8), 5 μM FTC (FTC; n = 8), or 1 μM Ko123 (n = 8). *B*: the cell monolayers were incubated at 37°C for 30 min with ^{14}C-BT (10 μM), after which efflux of ^{14}C-BT into the extracellular medium was allowed for 20 min in the absence (control) or presence of 20 μM verapamil (VER; n = 12), 100 μM quinidine (QUIN; n = 12), 100 μM vinblastine (VINB; n = 12), 100 μM indomethacin (IND; n = 8), 500 μM probenecid (PROB; n = 8), 5 μM FTC (FTC; n = 7), or 1 μM Ko123 (n = 7). The results are shown as means ± SE. *Significant difference compared with control condition ($P < 0.05$).

Effect of BCRP Inhibitors and BT On Influx and Efflux of ^{3}H-FA in IEC-6 Cells

^{3}H-FA is a known BCRP substrate (5, 65). So, in this series of experiments, we compared the effects of BCRP inhibitors (FTC and Ko143) and BT on ^{3}H-FA influx and efflux in IEC-6 cells.

As can be seen in Fig. 2*A*, Ko143 (1 μM) and BT (100 μM) were able to significantly increase influx of ^{3}H-FA (by 10–15%), and both BCRP inhibitors and BT significantly decreased (by 10–20%) ^{3}H-FA efflux (Fig. 2*B*). Overall, these results show that the effect of BT and BCRP inhibitors on ^{3}H-FA influx and ^{3}H-FA efflux in IEC-6 cells is very similar.

Effect of BCRP Inhibitors On Influx and Efflux of ^{14}C-BT in MDA-MB-231 Cells

Next, we investigated the effects of BCRP inhibitors (FTC and Ko143) on ^{14}C-BT influx and efflux from MDA-MB-231 cells. MDA-MB-231 cells are highly metastatic tumorigenic human breast cancer cells expressing high levels of hBCRP (13, 72). We verified that BCRP inhibitors increased ^{14}C-BT uptake (by 22–29%) and decreased ^{14}C-BT efflux (by 40%) (Fig. 3).

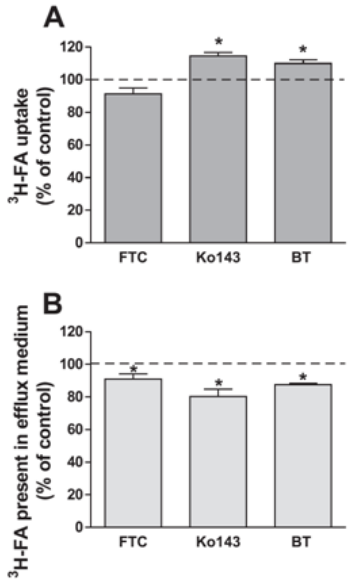


Fig. 2. Effect of breast cancer resistance protein (BCRP) inhibitors and of BT on the apical uptake (*A*) and efflux (*B*) of ^{3}H-FA by IEC-6 cells. *A*: the cell monolayers were incubated at 37°C for 3 min with ^{3}H-FA (10 nM) in the absence (control) or presence of 5 μM FTC (FTC; n = 13), 1 μM Ko143 (Ko143; n = 7), or 100 μM BT (BT; n = 10). *B*: the cell monolayers were incubated at 37°C for 30 min with ^{3}H-FA (10 nM), after which efflux of ^{3}H-FA into the extracellular medium was allowed for 20 min in the absence (control) or presence of 5 μM FTC (FTC; n = 13), 1 μM Ko143 (Ko143; n = 5), or 100 μM BT (BT; n = 6). The results are shown as means ± SE. *Significant difference compared with control condition ($P < 0.05$).

Effect of Inhibitors of ABC Transporters On Influx and Efflux of ^{14}C-BT in Caco-2 Cells and IEC-6 Cells Previously Exposed to BT

Exposure of many CRC cell lines to BT inhibits cellular proliferation and induces cellular differentiation and apoptosis, thus reducing cellular growth rate (29, 33). However, some CRC cells escape this effect of BT and become resistant to this agent, as evidenced by the existence of BT-resistant cell lines (34, 42). Furthermore, exposure to BT is known to increase MDR1 gene expression in small cell lung carcinoma (17), thyroid carcinoma (43), and CRC cell lines (44, 46). Given the important role of BT at the colonic epithelial level, identification of the mechanisms responsible for the acquisition of resistance to BT is of great potential value. So we next decided to evaluate the effect of ABC transporter inhibitors on the influx and efflux of ^{14}C-BT in Caco-2 and IEC-6 cells previously treated for 48 h with 2 mM BT.

In BT-treated Caco-2 cells, indomethacin (100 μM) and probenecid (500 μM) decreased (by 15–20%), whereas Ko143 increased (by 10%), the initial rates of ^{14}C-BT uptake (Fig. 4*A*). In relation to ^{14}C-BT efflux, it was significantly decreased (by 13%) by FTC (5 μM) (Fig. 4*B*). So, in BT-treated Caco-2 cells, there is no involvement of MDR1 and MRPs in ^{14}C-BT efflux, but, based on the FTC and Ko143 results, we can suggest an increase in BCRP-mediated ^{14}C-BT transport in BT-treated cells.

In BT-treated IEC-6 cells, ^{14}C-BT initial rates of uptake were increased by quinidine (100 μM), FTC (5 μM), and Ko143 (1 μM), and reduced by indomethacin (100 μM) (Fig. 4*C*). In relation to ^{14}C-BT efflux, it was significantly decreased (by 11%) in the presence of vinblastine (100 μM) (Fig. 4*D*). So, in BT-treated IEC-6 cells, there is no involvement of MDR1 and MRPs in ^{14}C-BT efflux. Moreover, the results obtained with the BCRP inhibitors FTC and Ko143, as a whole, suggest a slight decrease in BCRP-mediated ^{14}C-BT efflux in BT-treated IEC-6 cells. This conclusion is based in their discrepant effect on influx (inhibition) and efflux (no effect) of ^{14}C-BT, which in our opinion may be related to the fact that the effect of inhibitors is more evident in influx than in efflux experiments (see METHODS).

Effect of BT and Ko143 on Caco-2 and IEC-6 Cellular Viability, Proliferation, and Differentiation

The anticarcinogenic effect of BT depends on its intracellular concentration (11), which depends on efflux transport mechanisms. So, in this final series of experiments, we investigated whether inhibition of BCRP (with Ko143) would be able to modify the effect of BT on Caco-2 and IEC-6 cell viability, proliferation, and differentiation.

In relation to Caco-2 cells, an exposure to 5 mM BT for 48 h was chosen based on previous experiments from our group (24). As to IEC-6 cells, the effect of a 48-h treatment of these cells with increasing concentrations of BT on cell proliferation

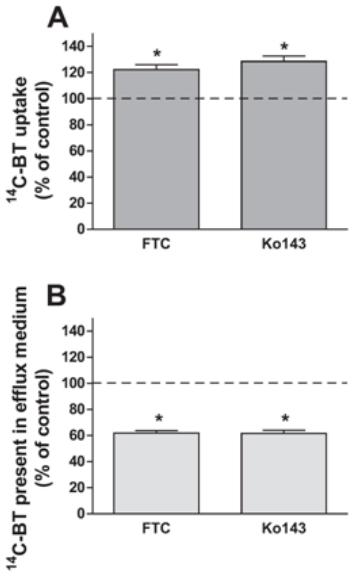


Fig. 3. Effect of BCRP inhibitors upon the apical uptake (*A*) and efflux (*B*) of ^{14}C-BT by MDA-MB-231 cells. *A*: the cell monolayers were incubated at 37°C for 3 min with ^{14}C-BT (10 μM) in the absence (control) or presence of 5 μM FTC (FTC; n = 8) or 1 μM Ko123 (Ko143; n = 8). *B*: the cell monolayers were incubated at 37°C for 30 min with ^{14}C-BT (10 μM), after which efflux of ^{14}C-BT into the extracellular medium was allowed for 20 min in the absence (control) or presence of 5 μM FTC (FTC; n = 8) or 1 μM Ko143 (Ko143; n = 8). The results are shown as arithmetic means ± SE. *Significant difference compared with control condition ($P < 0.05$).

was assessed in preliminary experiments. On the basis of the results obtained (see Fig. 6*A*), we selected also 5 mM BT for further experiments with these cells. As mentioned in METHODS, this concentration of BT is well within the physiological range of concentrations in human colonic lumen.

The effect of BT (5 mM) alone or in combination with Ko143 (100 nM) on Caco-2 cell viability, proliferation, and differentiation was first examined. As shown in Fig. 5, BT (5 mM) caused a significant decrease in cellular proliferation and viability and an increase in cell differentiation. This is in agreement with our laboratory's recently published results (24). On the other hand, Ko143 (100 nM) caused no changes in cellular viability, proliferation, and differentiation. When BT (5 mM) was combined with Ko143 (100 nM), a slight potentiation of the effect of BT on cell proliferation was observed. However, no change in the effect of BT on cell viability was observed, and the effect of BT on cell differentiation was even slightly decreased (Fig. 5).

In IEC-6 cells, BT (5 mM) caused a significant decrease in cellular proliferation and viability, and a very marked increase in cell differentiation (Fig. 6). To our knowledge, this is the first demonstration that BT affects the viability and proliferation of IEC-6 cells, although Fukushima et al. (21) already showed that BT induced IEC-6 cellular differentiation. Ko143 (100 nM) caused no changes in cellular viability and differentiation but produced a significant (18%) increase in cell proliferation. Interestingly enough, combination of BT with Ko143 did not potentiate the effect of BT on cellular viability and differentiation but caused a significant potentiation of the effect of BT on cell proliferation (Fig. 6).

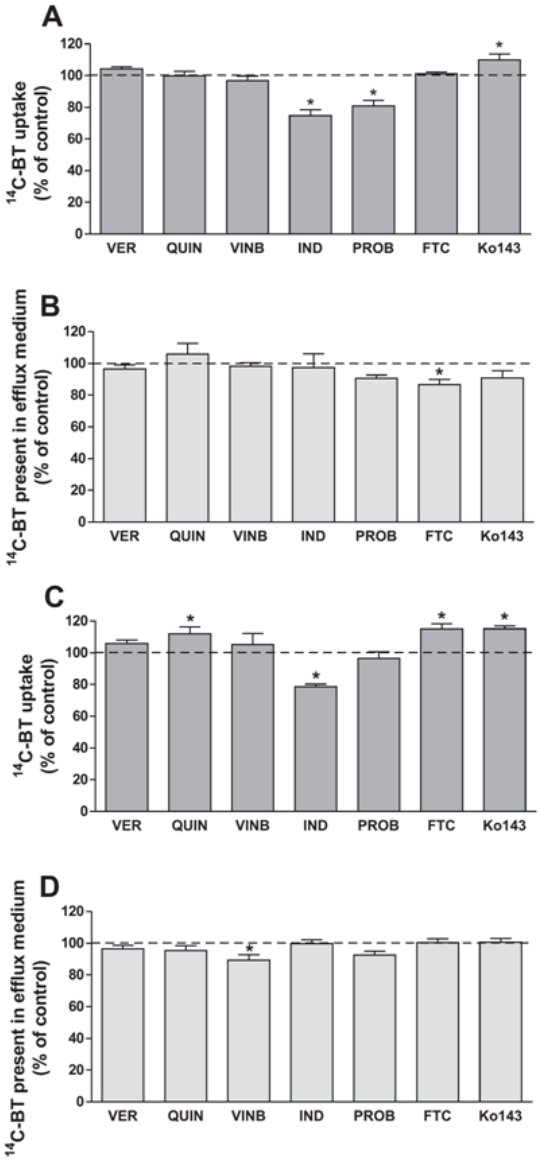


Fig. 4. Effect of various compounds on the apical uptake (*A*, *C*) and efflux (*B*, *D*) of ^{14}C-BT by 7-day-old Caco-2 cells (*A*, *B*) and IEC-6 cells (*C*, *D*) previously treated for 48 h with 2 mM BT. *A* and *C*: the cell monolayers were incubated at 37°C for 3 min with ^{14}C-BT (10 μM) in the absence (control) or presence of 20 μM verapamil (VER; $n = 8$), 100 μM quinidine (QUIN; $n = 8$), 100 μM vinblastine (VINB; $n = 12$), 100 μM indomethacin (IND; $n = 8$), 500 μM probenecid (PROB; $n = 8$), 5 μM FTC (FTC; $n = 8$), or 1 μM Ko123 ($n = 12$). *B*: the cell monolayers were incubated at 37°C for 30 min with ^{14}C-BT (10 μM), after which efflux of ^{14}C-BT into the extracellular medium was allowed for 5 min (Caco-2 cells) or 20 min (IEC-6 cells) in the absence (control) or presence of 20 μM verapamil (VER; $n = 8$), 100 μM quinidine (QUIN; $n = 8$), 100 μM vinblastine (VINB; $n = 8$), 100 μM indomethacin (IND; $n = 8$), 500 μM probenecid (PROB; $n = 12$), 5 μM FTC (FTC; $n = 12$), or 1 μM Ko123 ($n = 12$). The results are shown as arithmetic means ± SE. *Significant difference compared with control condition ($P < 0.05$).

DISCUSSION

The aim of this work was to investigate the putative involvement of members of the ABC superfamily of transporters on the handling of the histone deacetylase inhibitor BT in intestinal epithelial cells. We focused our research on MDR1, MRPs, and BCRP, because these members of the ABC family of transporters are present in high levels in the colon (see Introduction). We decided to investigate the involvement of efflux transporters in BT handling in a colon adenocarcinoma cell line, the Caco-2 cells (15, 55), and a non-tumoral epithelial intestinal cell line, the IEC-6 cells (69), because comparison between a carcinogenic and a noncarcinogenic cell line seemed interesting in the context of a possible distinct effect of BT in these cells.

Our results demonstrate that, both in 7-days-old and well differentiated (21-days-old) Caco-2 cells, there is no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in ^{14}C-BT efflux. In contrast, in IEC-6 cells, no involvement of MDR1 and MRPs in^{14}C-BT efflux was found, but we demonstrated the involvement of BCRP in ^{14}C-BT efflux, because both FTC and Ko143 increased uptake and decreased efflux of ^{14}C-BT.

The hypothesis that BT is indeed a BCRP substrate was further investigated. First, we demonstrated mRNA expression

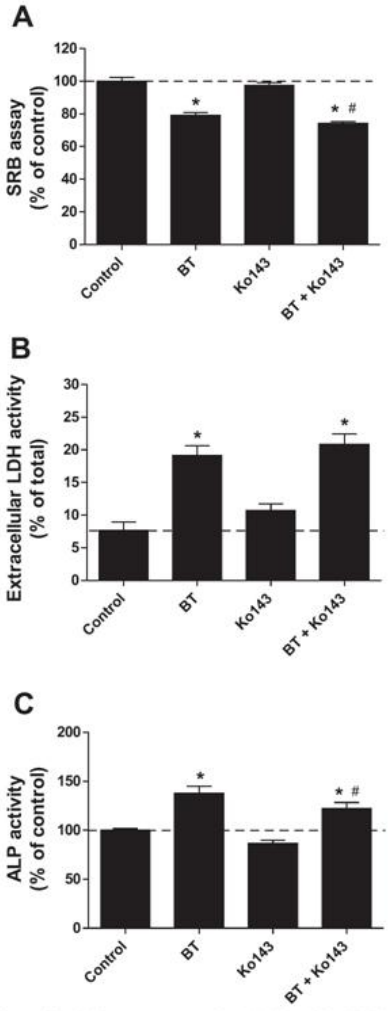


Fig. 5. Effect of a 48-h exposure to 5 mM BT, 100 nM Ko143, or a combination of both compounds (BT + Ko143) on Caco-2 cellular proliferation (*A*), viability (*B*), and differentiation (*C*). *A*: cellular proliferation was determined by quantification of whole cellular protein with SRB, as described in METHODS. Results are shown as absorvance (% of control; n = 24). *B*: cellular viability was determined by quantification of extracellular LDH activity, as described in METHODS. Results are shown as extracellular LDH activity (% of total LDH activity; n = 18). *C*: cell differentiation was determined by quantification of alkaline phosphatase (ALP) activity, as described in METHODS. Results are shown as nmol p-nitrophenol·min^{-1}·mg protein^{-1} (% of control; n = 24). Results are presented as arithmetic means + SE. *Significantly different from control ($P < 0.05$). #Significantly different from BT ($P < 0.05$).

of rBCRP in IEC-6 cells. Then we demonstrated that BT, similar to BCRP inhibitors, was able to increase the influx and decrease the efflux of the known BCRP substrate ^{3}H-FA (5, 65) in IEC-6 cells. Finally, we demonstrated that BCRP inhibitors were able to increase ^{14}C-BT influx and decrease ^{14}C-BT efflux in another BCRP-expressing cell line, the MDA-MB-231 cell line, which expresses high levels of functional hBCRP (13, 72). Moreover, these last results demonstrate that BT is also a substrate of hBCRP.

Although we have convincingly shown that BT is a BCRP substrate, we have shown BCRP-mediated BT transport in IEC-6 cells but not in Caco-2 cells. We think this difference is due to different BCRP expression levels in these two cell lines. In normal human tissues, BCRP reveals high levels of expression in small intestine and colon, with levels of BCRP expression higher in duodenum and then decreasing along the intestinal tract (28). However, Taipalensuu et al. (61) have demonstrated that BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with human jejunum. Accordingly, BCRP mRNA and protein expression are also downregulated in CRC (26). On the other hand, the IEC-6 cell line is a nontumorigenic intestinal rat cell line, and the expression of BCRP in rat intestine is relatively higher in relation to human intestine (62). From the results of this study, we can speculate that downregulation of BCRP in CRC may explain or contribute to the fact that tumor cells are more sensitive to the effect of BT than normal cells.

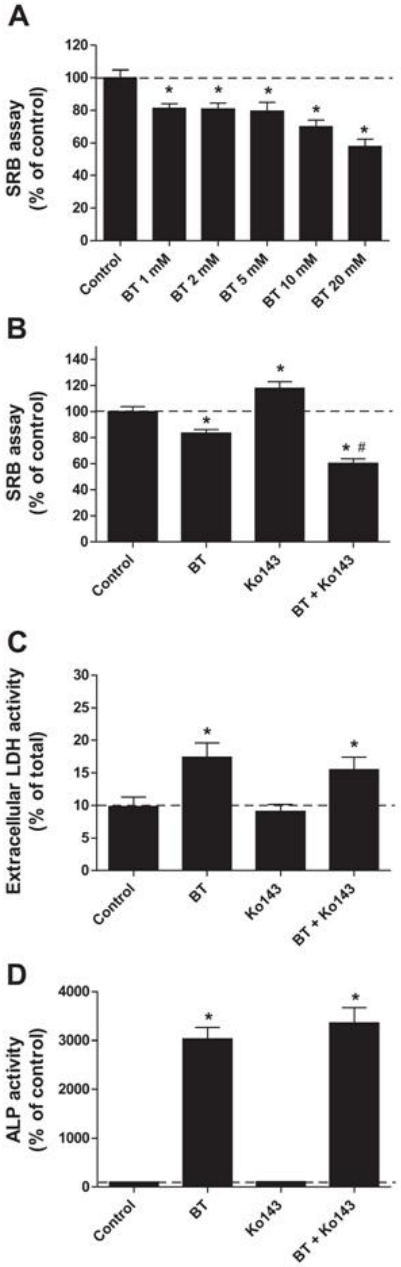
As stated in RESULTS, some CRC cells escape the anticarcinogenic effect of BT and become resistant to this agent, as evidenced by the existence of BT-resistant cell lines. Also, BT is known to increase MDR1 gene expression in some other cancer cell types. Given the important role of BT at the colonic epithelial level, identification of the mechanisms responsible for the acquisition of resistance to BT is of great potential value. So we also decided to evaluate the effect of ABC transporter inhibitors on the influx and efflux of ^{14}C-BT in Caco-2 and IEC-6 cells previously treated for 48 h with 2 mM BT.

In BT-treated Caco-2 cells, and similar to control cells, there is no involvement of MDR1 and MRPs in ^{14}C-BT transport. This lack of effect of BT treatment in inducing MDR1 and MRPs in Caco-2 cells is in agreement with a previous report (10). On the other hand, treatment with BT seems to increase the expression of BCRP. Recently, it was demonstrated that some HDAC increase BCRP expression in cancer cell lines (32, 54, 63). Different results were obtained in IEC-6 cells, where treatment with BT did not induce MDR1, MRPs, and BCRP. On the contrary, the results obtained with FTC and Ko143 suggest a slight decrease in BCRP-mediated ^{14}C-BT transport in BT-treated IEC-6 cells. In summary, efflux of ^{14}C-BT from BT-treated Caco-2 cells and IEC-6 cells seems to occur mainly via BCRP.

As mentioned before, exposure of many tumoral cell lines to BT inhibits cellular proliferation and induces cellular differentiation and apoptosis, thus reducing cellular growth rate. This anticarcinogenic effect of BT depends on its intracellular concentration (11), which is obviously dependent on efflux transport mechanisms. So, in the last part of this work, we decided to investigate whether inhibition of BCRP would affect the response of the cells to BT. Because BCRP seems to be involved in BT efflux in both BT-treated Caco-2 and IEC-6 cells, we tested the effect of BT in conjunction with an inhibitor of BCRP (Ko143) on viability, proliferation, and differentiation in both cell lines. We verified that, in both Caco-2 and IEC-6 cells, combination of BT (5 mM) with Ko143 (100 nM) did not potentiate the effect of BT on cell

viability and differentiation but potentiated the effect of BT on cell proliferation.

In summary, our results strongly support the conclusion that BT is a BCRP substrate (Fig. 7). Several lines of evidence support this conclusion. First, BCRP inhibitors reduced



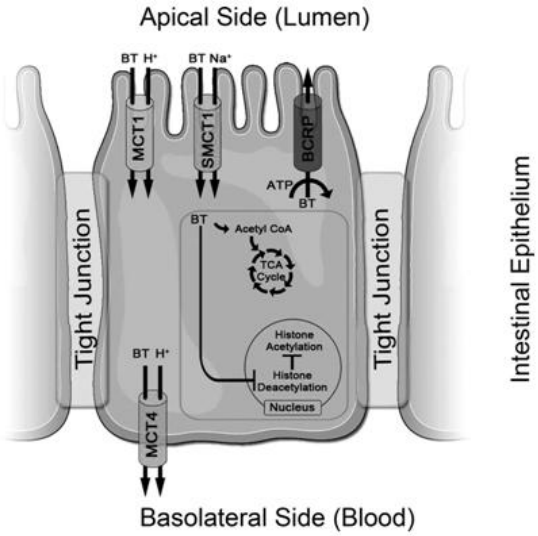


Fig. 7. Proposed model of expression and function of BT transporters and its main intracellular targets in colonocytes, including its metabolism in the Krebs cycle and its effect on histone acetylation. Carriers such as MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane (7, 26, 45). BCRP (gene name ABCG2) is an ATP-dependent efflux transporter for BT at the apical membrane (present results). At the basolateral membrane, efflux of BT occurs via MCT4 (monocarboxylate transporter 4, gene name SLC16A3) (21).

^{14}C-BT efflux in IEC-6 cells, and IEC-6 cells were found to express rBCRP mRNA. Second, both BT and BCRP inhibitors significantly decreased the efflux of the known BCRP substrate ^{3}H-FA in IEC-6 cells. Third, BCRP inhibitors reduced ^{14}C-BT efflux in the BCRP-expressing MDA-MB-231 cell line. So the interaction between BT and BCRP does not seem to be IEC-6 cell-specific, and BT appears to be a substrate of both rat and human BCRP. Finally, although BCRP is not involved in ^{14}C-BT efflux in Caco-2 cells, treatment of these cells with BT induced BCRP-mediated ^{14}C-efflux. So BT appears to induce BCRP expression in Caco-2 cells.

The results of this study, showing for the first time that BT is a BCRP substrate, are very important in the context of the high levels of BCRP expression in the human colon (28, 61), where high concentrations of BT are also present. Given the

Fig. 6. Effect of a 48-h exposure to increasing concentrations of BT (1, 2, 5, 10, or 20 mM) on IEC-6 cell proliferation (*A*) and effect of a 48-h exposure to 5 mM BT, 100 nM Ko143, or a combination of both compounds (BT + Ko143) on IEC-6 cellular proliferation (*B*), viability (*C*), and differentiation (*D*). *A* and *B*: cellular proliferation was determined by quantification of whole cellular protein with SRB, as described in METHODS. Results are shown as absorvance (% of control; $n = 12$). *C*: cellular viability was determined by quantification of extracellular LDH activity, as described in METHODS. Results are shown as extracellular LDH activity (% of total LDH activity; $n = 30$). *D*: cell differentiation was determined by quantification of alkaline phosphatase (ALP) activity, as described in METHODS. Results are shown as nmol *p*-nitrophenol·min^{-1}·mg protein^{-1} (% of control; $n = 24$). Results are presented as arithmetic means + SE. *Significantly different from control ($P < 0.05$). #Significantly different from BT ($P < 0.05$).

important physiological role of BT at that level, the interaction of BT with BCRP and other BCRP substrates/inhibitors is indeed of major importance, for instance, in the context of carcinogenesis and inflammatory bowel disease (ulcerative colitis and Crown disease). Two examples of such interaction are given next. First, BCRP acts as an efflux transporter for various anticancer agents including 5-fluorouracil, methotrexate, mitoxantrone, anthracyclines, daunorubicin, doxorubicin, topotecan, diflomotecan, irinotecan, tyrosine kinase inhibitors (e.g., imatinib and gefitinib), and nucleoside analogs (49, 52). Thus it prevents the buildup of high intracellular concentrations of such anticancer agents and decreases their cytotoxic effects. It is interesting to speculate that BT, by competing with these agents for BCRP, might increase the intracellular concentration of these anticancer agents and thus improve their therapeutic efficacy. On the other hand, it is also interesting to speculate that some anticancer agents (e.g., tyrosine kinase inhibitors), by competing with BT for BCRP, cause an increase in the intracellular concentration of BT. This might be important in the context of the known anticarcinogenic effect of BT. Interestingly, a synergism between the anticarcinogenic effect of BT and 5-fluorouracil (4), the nucleoside analog 5-aza-2′-deoxycytidine (6), doxorubicin (41, 43), and tyrosine kinase inhibitors (erlotinib and gefitinib) (38) exists. The combination of BT and these anticancer agents might have clinical implications for CRC therapy. Second, BCRP was also reported to transport folates such as FA (5, 65). This folate exporter function is consistent with BCRP having a possible role in the maintenance of cellular folate homeostasis (35). So we can also speculate that inhibition of BCRP-mediated folate efflux by BT may contribute to its protective role against CRC by increasing the intracellular concentration of folate, which is known to decrease the risk for developing CRC (39). It would be very interesting to test both these hypotheses in the near future in the human colon (e.g., by using human colonic biopsies).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Modulation of BT transport and of its anticarcinogenic effect

IV - Effect of some natural mineral waters in nutrient uptake by Caco-2 cells

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Original Communication

Effect of Some Natural Mineral Waters in Nutrient Uptake by Caco-2 Cells

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Abstract: We studied the effect of some mineral waters and some of their constituents on the apical uptake of ^{14}C-butyrate (^{14}C-BT) and ^{3}H-O-methyl-D-glucose (^{3}H-OMG) by Caco-2 cells. Uptake of ^{14}C-BT increased after a 20-minute exposure to 1 % (v/v) distilled water, and, compared to distilled water, it was decreased by Pedras Salgadas® 1 % (v/v) and Melgaço® 5 % (v/v), and increased by Vidago® 5 % (v/v). Moreover, it increased after a 48-hour exposure to Vidago® or Melgaço® waters (5 % (v/v)). Also, uptake of ^{14}C-BT was reduced after a 20-minute exposure to $MgCl_2$, $MgSO_4$, or $CaCl_2$. Uptake of ^{3}H-OMG was reduced after a 20-minute exposure to Melgaço® water [1 % (v/v)], when compared to distilled water. Also, a 48-hour exposure to Pedras Salgadas® or Melgaço® water (5 % (v/v)) increased and decreased uptake, respectively. Finally, uptake of ^{3}H-OMG decreased after a 20-minute exposure to $MgSO_4$ or NaF. In conclusion, uptake of ^{14}C-BT and ^{3}H-OMG by Caco-2 cells is differently modulated by distinct mineral waters.

Key words: butyrate; Caco-2 cells; glucose; uptake; natural mineral waters.

Introduction

Natural mineral waters are waters of underground origin, protected from contamination and microbiologically wholesome; they present a peculiar and constant chemical composition, and have a favorable effect on health. Natural mineral waters are characterized by purity at the source, mineral content, trace elements and other constituents, and conservation and healing properties recognized by clinical and pharmacological trials [1].

Based on biological activity, natural mineral waters can be classified as diuretic waters, cathartic waters, or waters with antiphlogistic properties [1]. A number of studies have suggested that natural mineral waters are valid tools in the treatment of a large number of disorders including gastrointestinal [2, 3], renal [4], cardiovascular and cerebrovascular [5–13], neurologic [14], obstetric-gynecologic [1, 13], hematologic, *rheumatic, dermatological,* and respiratory [1]. Moreover, natural mineral waters seem to possess anti-oxidant properties [6, 15, 16]. However, very little is known concerning the effect of natural mineral waters upon the intestinal absorption of nutrients. Thus, the aim of this study was to investigate the effect of natural mineral waters on the intestinal absorption of two different nutrients, glucose and butyrate.

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Short-chain fatty acids (SCFAs; acetate, propionate, and butyrate) are main end-products of anaerobic bacterial fermentation of dietary fiber within the human colon. Among SCFAs, butyrate (BT) plays a key role in colonic epithelium homeostasis because it has multiple important roles at that level, including (1) being the main energy source for the colonocyte [17–19]; (2) inhibiting colon carcinogenesis [20–23]; (3) promoting growth and proliferation of normal colonic epithelial cells [24–26]; (4) stimulating fluid and electrolyte absorption [27–29]; (5) inhibiting colon inflammation and oxidative stress; and (6) improving the colonic defense barrier function [30, 31].

BT and other SCFAs are transported into colonic epithelial cells by passive, non-ionic diffusion of the undissociated SCFA form, and by specific carrier-mediated transport of SCFA anions that seems to include: (1) a SCFA/HCO_3^- exchanger, (2) an electroneutral H^+-coupled monocarboxylate transporter (MCT) [32–34], and (3) an electrogenic Na^+-coupled transporter for monocarboxylates (SMCT1) [35]. One of the proposed beneficial effects of BT on human intestinal health is the prevention and inhibition of colon carcinogenesis. In this context, it is very interesting to verify that both MCT1 [36–39] and SMCT1 [35, 40] were recently proposed to function as tumor suppressors. So, both SMCT- and MCT1-mediated transport of BT into colonocytes is fundamental for the regulation of cell homeostasis. However, very little is known concerning regulation of BT transport: MCT1-mediated transport at the intestinal level is up-regulated by its substrate, BT [42, 43]; enhanced by leptin [44], phorbol 12-myristate 13-acetate [45], caffeine, and acetylsalicylic acid [46]; and inhibited by enteropathogenic *E. coli* [47], interferon-γ, tumor necrosis factor-α [48], theophylline, tetrahydrocannabinol, MDMA (ecstasy), acetaldehyde, and indomethacin [46].

Glucose derived from the diet is absorbed in the early- and mid-small intestine by the mature enterocytes on the upper third of the villi [49]. According to the "classical model of sugar absorption," glucose is actively taken up into the enterocytes from the intestinal lumen by a high-affinity, Na^+-dependent, and phloridzin-sensitive glucose co-transporter (SGLT1) located in the brush border and is then passively released from the enterocytes into the circulation via a Na+-independent glucose transporter 2 (GLUT2) present in the basolateral membrane. More recently, evidence for the presence of a functional GLUT2

Table I: Chemical composition of the natural mineral waters Pedras Salgadas®, Vidago®, and Melgaço®

	Pedras Salgadas®	Vidago®	Melgaço®
Total dissolved solids (mg/L)	1973	1870	711
Electrical conductivity (μS/cm)	2260	2370	909
pH	6.16	6.16	5.72
Free CO_2 (g/L)	3.9	3.4	5.1
Na^+ (mg/L)	641	627	82.2
K^+ (mg/L)	31.2	46.8	3.4
Ca^{2+} (mg/L)	106	82	137
Mg^{2+} (mg/L)	26.1	15.2	30.3
Li^+ (mg/L)	2.1	2.9	0.61
Mn^{2+} (mg/L)	0.215	0.224	0.245
Sr^{2+} (mg/L)	0.54	0.49	2.3
NH_4^+ (mg/L)	< 0.05	0.35	< 0.05
F^- (mg/L)	1.5	1.1	1.1
Cl^- (mg/L)	31.2	34.8	12.1
HCO_3^- (mg/L)	2135	2013	763
SO_4^{2-} (mg/L)	5.3	6.3	6.1
SiO_2 (mg/L)	60.4	46.4	53.8
NO_3^- (mg/L)	0.18	0.25	< 0.12

transporter at the brush-border membrane, participating also in the intestinal absorption of glucose, has been obtained [49–54].

The aim of this study was to investigate the influence of some natural mineral waters on the intestinal absorption of butyrate and glucose. Knowledge on this subject seems important given the fact that ingestion of bottled mineral water has been growing in recent years and the important physiological roles played by glucose and butyrate. In order to investigate this, we determined the effect of three distinct natural mineral waters (Pedras Salgadas®, Vidago®, and Melgaço®) upon the apical uptake of ^{14}C-BT and ^{3}H-O-methyl-D-glucose (OMG; a glucose analogue) by Caco-2 cells. The Caco-2 cell line forms monolayers of viable and polarized intestinal epithelial cells that mimic the intestinal absorptive epithelium. This cell line exhibits several characteristics, such as carrier-mediated transport systems and membrane enzyme activities, of small intestinal epithelial cells, and is considered an intestinal epithelial absorption model [55, 56].

Methods and materials

Caco-2 cell culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage numbers 28–35 and 63–78. The cells were maintained in a humidified atmosphere of 5 % CO_2-95 % air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 5.55 mM glucose and supplemented with 15 % fetal calf serum, 25 mM HEPES, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all from Sigma). Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25 % trypsin-EDTA, 5 minutes, 37 °C), split 1:3, and sub-cultured in plastic culture dishes (21-cm^2; 60 mm; TPP®, Trasadingen, Switzerland). For uptake studies, Caco-2 cells were seeded (seeding density of 0.65×10^5 cells/cm^2) on 24-well plastic cell culture clusters (2-cm^2; 16 mm; TPP®), and the experiments were performed 8–11 days after the initial seeding. The cell medium was free of fetal calf serum for 24 hours before the experiments.

Determination of ^{14}C-BT and ^{3}H-OMG uptake by Caco-2 cells

Uptake experiments were performed with Caco-2 cells incubated in glucose-free HEPES buffered saline (containing, in mM: 140 NaCl, 5 KCl, 2.5 $MgSO_4$, 1 $CaCl_2$, 20 HEPES; pH 6.5 and 7.5 for ^{14}C-BT and ^{3}H-OMG experiments, respectively). Initially, the culture medium was aspirated and the cells were washed with 0.3 mL buffer at 37 °C. Then, the cell monolayers were pre-incubated for 20 minutes in 0.3 mL buffer at 37 °C. Uptake was initiated by the addition of 0.3 mL medium at 37 °C containing ^{14}C-BT (10 µM or 20 mM) or ^{3}H-OMG (10 µM or 5 mM). Incubation was stopped after 3 minutes (except in the time-course experiments) by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.3 mL ice-cold buffer. The cells were then solubilized with 0.3 mL 0.1 % (v/v) Triton X-100 (in 5 mM Tris.HCl, pH 7.4), and placed at 37 °C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Treatment of the cells with natural mineral waters

Acute and sub-acute treatment. The acute and sub-acute effect of natural mineral waters on ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) uptake was tested by pre-incubating (20 minutes or 2 hours for acute and sub-acute treatment, respectively) and incubating cells (3 minutes) with ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) in the presence of the waters [1, 2, or 5 % (v/v)], which were added to the buffer, or in the presence of the corresponding amount of distilled water [1, 2, or 5 % (v/v]; control). The mineral waters were degassed before being added to the buffer; in this way, no significant change in buffer pH occurred.

Chronic effect. The chronic effect of natural mineral waters on ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) uptake was tested by cultivating 6- to 9-day-old cell cultures (90–95 % confluence) in culture medium in the presence of the natural mineral water [5 % (v/v)] or distilled water [5 % (v/v); control]. The mineral waters were not degassed before being added to the culture medium, because their inclusion did not cause a significant change in culture medium pH. The medium was renewed daily, and the transport experiments were performed after 48 hours. The transport experiments were identical to the experiments described above, except that there was no pre-incubation period, and cells were incubated with ^{14}C-BT or ^{3}H-OMG (3 minutes) in the absence of distilled or natural mineral waters.

Treatment of the cells with mineral ions

The acute effect of mineral ions on ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) uptake was tested by pre-incubating (20 minutes) and incubating cells (3 minutes) with ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) in the absence or presence of the mineral ions.

Protein determination

The protein content of cell monolayers was determined as described by Bradford [57], using human serum albumin as standard.

Calculation and statistics

For the analysis of the time course of ^{14}C-BT or ^{3}H-OMG uptake, the parameters of equation (1) were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method [58].

$$A(t)=k_{in}/k_{out}\,(1-e^{-k_{out}\cdot t}) \quad (1)$$

A(t) represents the accumulation of ^{14}C-BT or ^{3}H-OMG at time t, k_{in} and k_{out} the rate constants for inward and outward transport, respectively, and t the incubation time. A_{max} corresponds to the accumulation (A(t)) at steady-state ($t\rightarrow\infty$). K_{in} is given in pmol/mg protein.min and k_{out} in min^{-1}. In order to obtain clearance values, k_{in} was converted to µL/mg protein.min.

Arithmetic means are given with SEM, and geometric means are given with 95 % confidence limits. The n refers to the total number of replicates, which were obtained over 2–3 experiments conducted in different days. Statistical significance of the difference between various groups was evaluated by the Student's *t*-test. Differences were considered to be significant when $p < 0.05$.

Materials

^{14}C-BT (*n*-butyric acid, sodium salt, [1-^{14}C]; specific activity 30–60 mCi/mmol, ^{3}H-O-methyl-glucose (3-O-[methyl-^{3}H]; specific activity 40 Ci/mmol) (Biotrend Chemikalien GmbH, Köln, Germany), antibiotic/antimycotic solution (100 units mL^{-1} penicillin; 100 µg mL^{-1} streptomycin, and 0.25 µg mL^{-1} amphotericin B), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), sodium butyrate, $MgCl_2 \bullet 6H_2O$, $CaCl_2 \bullet 2H_2O$, LiCl, $MgSO_4 \bullet 7H_2O$, NaF, TRIS [tris-(hydroxymethyl)-aminomethane hydrochloride], trypsin-EDTA solution (Sigma, St. Louis, MO, USA); DMSO (dimethylsulfoxide), Triton X-100 (Merck, Darmstadt, Germany); fetal calf serum (Invitrogen Corporation, Carlsbad, CA, USA). The natural mineral waters to be tested (Pedras Salgadas®, Vidago® and Melgaço®) were kindly donated by iBesa (Instituto de Bebidas e Saúde, S. Mamede Infesta, Portugal).

Results

Time-dependence of nutrient apical uptake in Caco-2 cells

^{14}C-BT uptake

As shown in Figure 1, Caco-2 cells accumulated 10 µM or 20 mM ^{14}C-BT in a time-dependent way. Uptake of both 10 µM and 20 mM ^{14}C-BT was found to be

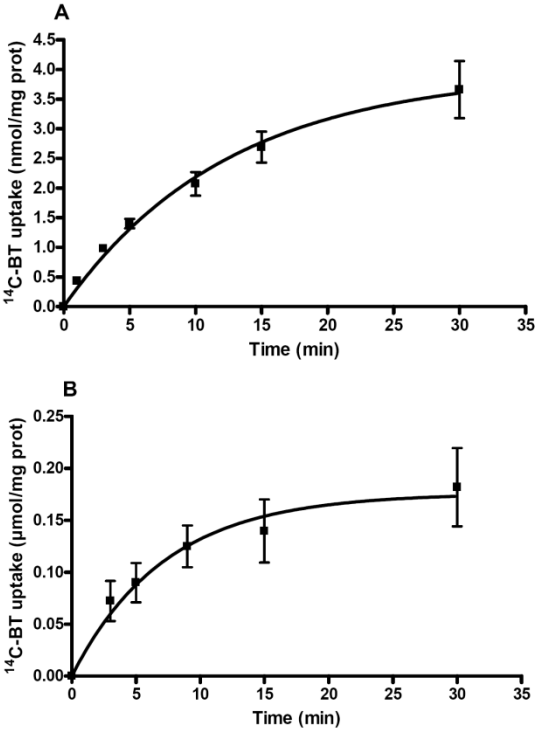

Figure 1: Time-course of ^{14}C-BT apical uptake in Caco-2 cells. Caco-2 cells were incubated at 37 °C with (**A**) 10 µM ($n = 4$), or (**B**) 20 mM ($n = 6–8$) ^{14}C-BT. Shown are arithmetic means ± SEM. The results shown in (**A**) were taken from [46].

linear with time for up to 3 minutes of incubation. So, in subsequent experiments, cells were incubated with 20 mM ^{14}C-BT for 3 minutes, in order to measure initial rates of ^{14}C-BT uptake.

^{3}H-OMG uptake

As shown in Figure 2, Caco-2 cells accumulated 10 μM or 5 mM ^{3}H-OMG in a time-dependent way, and uptake of both 10 μM and 5 mM ^{3}H-OMG was found to be linear with time for up to 3 minutes of incubation. So, in subsequent experiments, cells were incubated with 10 μM ^{3}H-OMG for 3 minutes, in order to measure initial rates of ^{3}H-OMG uptake.

Modulation of nutrient apical uptake in Caco-2 cells by natural mineral waters

In this series of experiments, the acute (20 minutes), sub-acute (2 hours) and chronic (48 hours) effects of three distinct natural mineral waters upon the apical uptake of ^{14}C-BT and ^{3}H-OMG by Caco-2 cells were investigated.

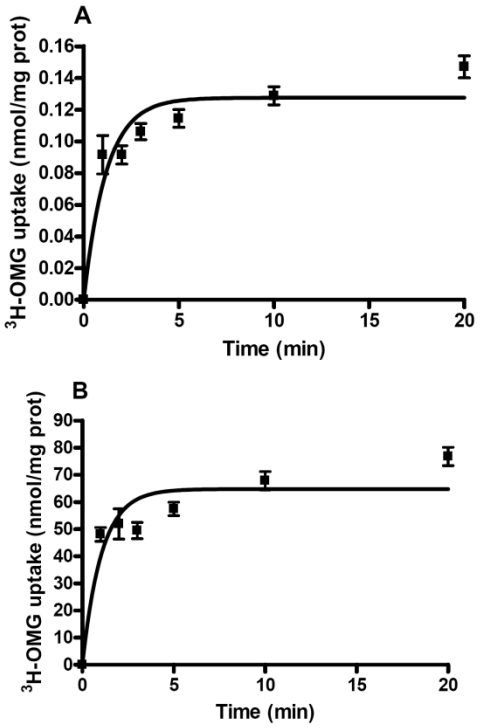

Figure 2: Time-course of ^{3}H-OMG apical uptake in Caco-2 cells. Caco-2 cells were incubated at 37 °C with (A) 10 μM (n = 4–6), or (B) 5 mM (n = 4–6) ^{3}H-OMG. Shown are arithmetic means±SEM.

^{14}C-BT uptake

Acute effect. Exposure of Caco-2 cells for 20 minutes to different concentrations of natural mineral waters [1, 2 and 5 % (v/v)] produced distinct results. The presence of the waters Pedras Salgadas® [1 % (v/v)] and Melgaço® [5 % (v/v)] reduced ^{14}C-BT uptake (by 16 and 16 %, respectively), when comparing with distilled water. On the other hand, Vidago® water [5 % (v/v)] caused a significant increase (of about 16 %) in ^{14}C-BT uptake. Interestingly, uptake of ^{14}C-BT in the presence of 1 % (v/v) distilled water was significantly higher (by about 16 %) than in control conditions (Figure 3).

Sub-acute-effect. When the exposure time was increased to 2 hours, none of the natural mineral waters, all tested at 5 % (v/v), showed any effect upon ^{14}C-BT uptake (Figure 4).

Chronic effect. Interestingly, Vidago® and Melgaço® waters caused an increase (of about 21 and 40 %, respectively) in ^{14}C-BT uptake (Figure 4).

^{3}H-OMG uptake

Acute effect. Exposure of Caco-2 cells for 20 minutes to different concentrations of natural mineral waters [1, 2 and 5 % (v/v)] showed no effect, apart from a decrease (of about 16 %) in uptake of this compound observed with Melgaço® water [1 % (v/v)] (Figure 5).

Sub-acute effect. When cells were exposed for 2 hours to the natural mineral waters [5 % (v/v)], no significant effect was found (Figure 6).

Chronic effect. Chronical (48 hours) uptake of ^{3}H-OMG was increased by Pedras Salgadas® mineral water (by about 35 %), and decreased (by about 10 %) by Melgaço® water (Figure 6).

Modulation of nutrient apical uptake in Caco-2 cells by mineral ions

In this series of experiments, the effect of some mineral ions present in the natural mineral waters was tested. For this series, cells were exposed for 20 minutes to buffers to which different salts ($MgCl_2$, $MgSO_4$, $CaCl_2$, LiCl, or NaF) were added. The concentrations of Mg^{2+}, Ca^{2+}, Li^{+}, or F^{-} added to the buffer are similar to those present in the natural mineral waters. For Cl^{-} and

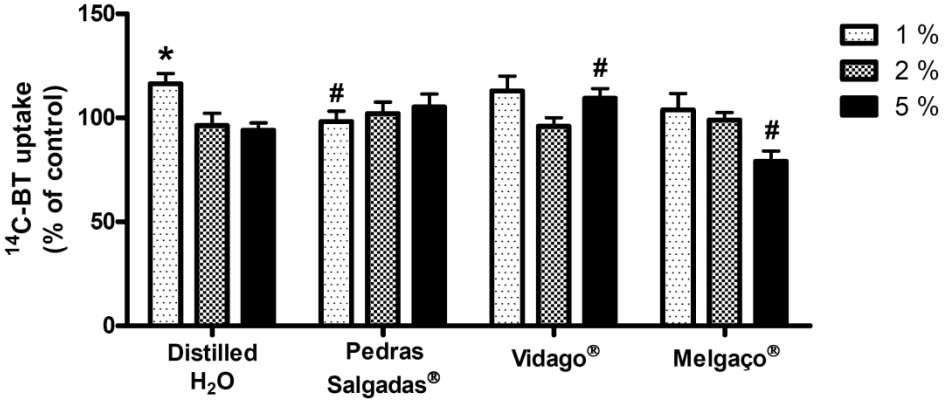

Figure 3: Acute effect (20 minutes) of natural mineral waters upon the apical uptake of 14C-BT by Caco-2 cells. Caco-2 cells were incubated with 14C-BT (20 mM) at 37°C for 3 minutes, and the effect of distilled water [1, 2 and 5 % (v/v); n = 8–13] and of the natural mineral waters Pedras Salgadas® [1, 2 and 5 % (v/v); n = 9], Vidago® [1, 2 and 5 % (v/v); n = 9], and Melgaço® [1, 2 and 5 % (v/v); n=9–13] were tested. Shown are arithmetic means±SEM. *Significantly different from control ($p < 0.05$) #Significantly different from distilled water ($p < 0.05$).

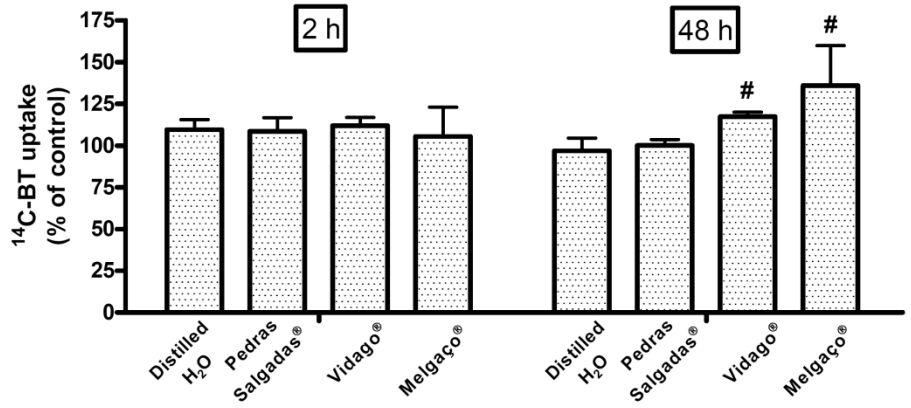

Figure 4: Sub-acute (2 hours) and chronic effect (48 hours) of natural mineral waters upon the apical uptake of 14C-BT by Caco-2 cells. Caco-2 cells were incubated with 14C-BT (20 mM) at 37°C for 3 minutes, and the effect of distilled water [5 % (v/v); n = 11] and of the natural mineral waters Pedras Salgadas® [5 % (v/v); n = 11], Vidago® [5 % (v/v); n = 8–11], and Melgaço® [5 % (v/v); n = 8–11] were tested. Shown are arithmetic means±SEM. *Significantly different from control ($p < 0.05$) #Significantly different from distilled water ($p < 0.05$).

SO_4^{2-}, their concentration in the buffer already largely exceeds the concentration present in the mineral waters.

^{14}C-BT uptake

Uptake of ^{14}C-BT (20 mM) by Caco-2 cells was significantly reduced in the presence of $MgCl_2$ (100 and 1000 mg/L Mg^{2+}), $MgSO_4$ (1000 mg/L Mg^{2+}), and $CaCl_2$ (100 and 1000 mg/L Ca^{2+}), to maximum values of 70, 71, and 85 % of control, respectively (Figure 7). However, uptake of this compound was not affected in the presence of LiCl or of NaF (0.1 to 10 mg/L) (Figure 7).

^{3}H-OMG uptake

Uptake of ^{3}H-OMG (10 µM) by Caco-2 cells was not changed in the presence of either $MgCl_2$, $CaCl_2$ (each at 100 and 1000 mg/L), or LiCl (0.1 to 10 mg/L). However, it was significantly reduced in the presence of $MgSO_4$

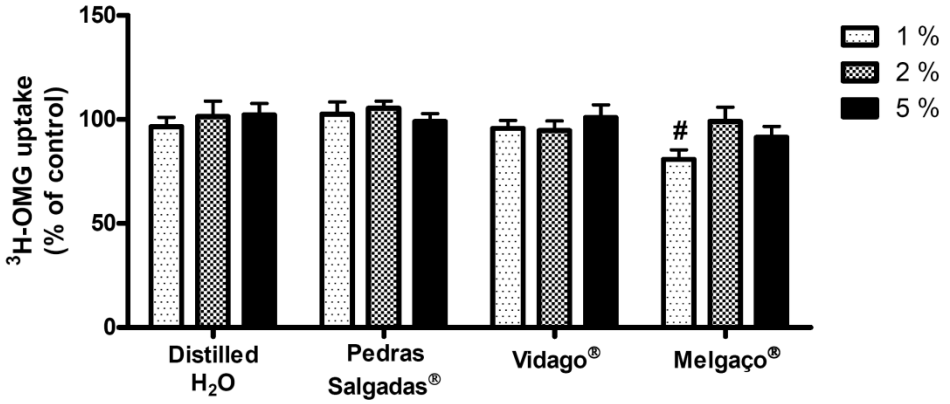

Figure 5: Acute effect (20 minutes) of natural mineral waters upon the apical uptake of ^{3}H-OMG by Caco-2 cells. Caco-2 cells were incubated with ^{3}H-OMG (10 μM) at 37ºC for 3 minutes, and the effect of distilled water [1, 2 and 5 % (v/v); *n*=9–11] and of the natural mineral waters Pedras Salgadas® [1, 2 and 5 % (v/v); *n*=9], Vidago® [1, 2 and 5 % (v/v); *n*=9–11], and Melgaço® [1, 2 and 5 % (v/v); *n*=9–11] were tested. Shown are arithmetic means±SEM. *Significantly different from control ($p<0.05$) #Significantly different from distilled water ($p<0.05$).

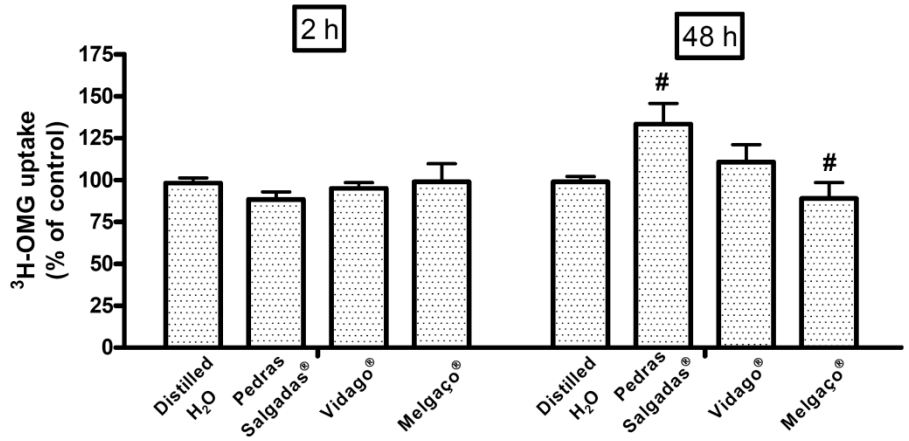

Figure 6: Sub-acute (2 hours) and chronic effect (48 hours) of natural mineral waters upon the apical uptake of ^{3}H-OMG by Caco-2 cells. Caco-2 cells were incubated with ^{3}H-OMG (10 μM) at 37ºC for 3 minutes, and the effect of distilled water [5 % (v/v); *n* = 8–12] and of the natural mineral waters Pedras Salgadas® [5 % (v/v); *n* = 8], Vidago® [5 % (v/v); *n* = 8], and Melgaço® (5 % (v/v); *n* = 8) were tested. Shown are arithmetic means ± SEM. *Significantly different from control ($p < 0.05$) #Significantly different from distilled water ($p < 0.05$).

(1000 mg/L) (to 84 % of control) and NaF (0.1 and 1 mg/L) (to a maximum of 83 % of control) (Figure 7).

Discussion

Water is the basic element of living beings, being involved in many body functions. Water homeostasis is essential for hydro-electrolytic balance, acid-base balance, and thermal balance, in addition to metabolic and plastic processes. Besides water, the ingredients of drinking water are also considered important to people's health (see Introduction). For the last 30 years, ingestion of bottled mineral water has been growing 7 % per year [59], with occidental European countries being the main producers and consumers [59, 60].

Although natural mineral waters are claimed to possess preventive and therapeutic effects in a number of pathologies (see Introduction), their effect upon the intestinal absorption of nutrients has not been assessed. The aim of this study was to investigate the

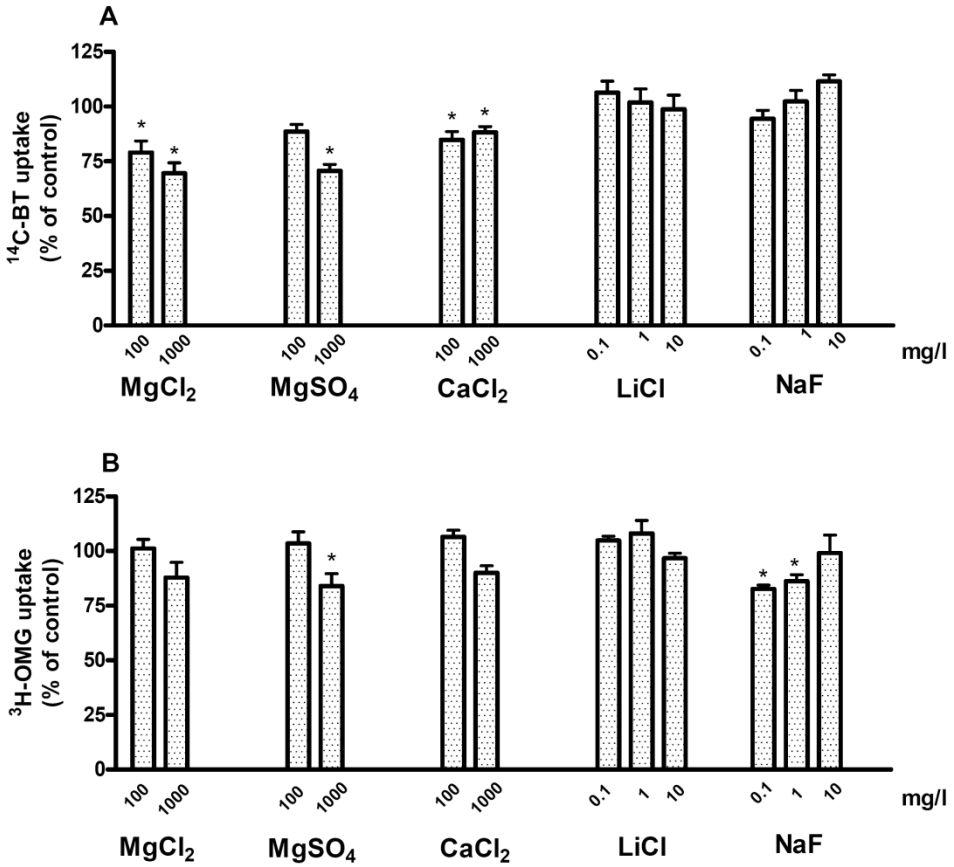

Figure 7: Effect of mineral ions upon the apical uptake of ^{14}C-BT (A) and ^{3}H-OMG (B) by Caco-2 cells. Caco-2 cells were incubated with ^{14}C-BT (20 mM) or ^{3}H-OMG (10 µM) at 37°C for 3 minutes, and the effect of $MgCl_2$ (100 or 1000 mg/L Mg^{2+}; $n = 6$), $MgSO_4$ (100 or 1000 mg/L Mg^{2+}; $n = 7$), $CaCl_2$ (100 or 1000 mg/L Ca^{2+}; $n = 6–9$), LiCl (0.1, 1 or 10 mg/L Li^{+}; $n = 6$), and NaF (0.1, 1 or 10 mg/L F^{-}; $n = 7$) were tested. Shown are arithmetic means ± SEM. *Significantly different from control ($p < 0.05$).

acute, sub-acute, and chronic effect of some natural mineral waters upon the intestinal absorption of glucose and BT. The acute, sub-acute, and chronic effects of the waters were tested because different mechanisms operate in these conditions, namely a change in the intrinsic activity of the transporters and in the amount of functionally active transporters present in the cell membrane, respectively [32, 34, 61].

According to the classification of the natural mineral waters based on dry residues at 180 °C, Pedras Salgadas® and Vidago® water are mineral-rich waters, while Melgaço® water is a medium-mineral content water. Moreover, according to the predominant chemical elements resulting in their biological and healing effects, Pedras Salgadas® and Vidago® waters are classified as sodium-bicarbonate-fluorate-carbonic waters, while Melgaço® water is a bicarbonate-fluorate-carbonic water [1].

Because BT plays an important role in maintenance of intestinal homeostasis, we decided to investigate the effect of mineral waters upon its cellular uptake. Previous works concluded that BT uptake by Caco-2 cells is mainly mediated by MCT1 [46, 62–64], which is highly expressed in these cells [66, 67].

Both acutely and chronically, Vidago® water increased ^{14}C-BT uptake, while Melgaço® water acutely decreased and chronically increased it, and Pedras Salgadas® acutely decreased uptake and chronically had no effect. Moreover, uptake of ^{14}C-BT was reduced after acute exposure to $MgCl_2$, $MgSO_4$, and $CaCl_2$.

It is well known that the causes of colorectal carcinoma (CCR) are multifactorial, and epidemiologic

evidence supports an association between dietary and other life style factors and the risk of CCR [65]. Our results, showing that chronic exposure to Melgaço® and Vidago® water increases Caco-2 uptake of ^{14}C-BT, suggest that chronic ingestion of these waters might possess a preventive effect against CCR. Recently, ingestion of chlorine-sulfur-bicarbonate mineral water was found to possess anti-oxidant effects [16], and ingestion of desalinated deep seawater was found to increase glutathione peroxidase activity [6]. Here, we show that natural mineral waters can have also a protective role against CCR by increasing epithelial uptake of BT.

Because glucose plays a major role as the main fuel in the body, and because blood levels of glucose, which are dependent on the intestinal absorption of this compound, play a key role in pathologies such as diabetes and obesity, it seemed important to investigate the effect of natural mineral waters upon its cellular uptake. OMG, a non-metabolized D-glucose analogue, is a substrate of both SGLT1 and GLUT2 at the intestinal level [66, 67], and in Caco-2 cells, where they are functionally present [66, 68, 69].

Both acutely and chronically, Melgaço® water decreased ^{3}H-OMG uptake, while chronic exposure to Pedras Salgadas® increased uptake. Moreover, uptake of ^{3}H-OMG was decreased after acute exposure to $MgSO_4$ and NaF.

The inhibitory effect of the Melgaço® water upon ^{3}H-OMG glucose suggests that ingestion of this mineral water may reduce the intestinal absorption of glucose, thus having beneficial effects on pathologies such as diabetes and obesity. This observation is most interesting when related to the fact that diabetes and obesity are both associated with an increased intestinal absorption of glucose and fructose, through increased expression of SGLT1, GLUT2, and the GLUT5 fructose transporter, and through increased Na^+/K^+ ATPase activity [49, 52, 70]. Moreover, based on the results obtained with chronic Melgaço® water upon ^{3}H-OMG and ^{14}C-BT uptake, we hypothesize that this water may be particularly useful in CCR prevention or treatment, as it increases ^{14}C-BT uptake while simultaneously decreasing ^{3}H-OMG uptake. This is because BT uptake is necessary for maintaining colonic epithelial homeostasis, and because glucose uptake and glucose transporter's expression increases, and simultaneously BT uptake and transporter's expression decreases, in colonic epithelial cells during transition from normality to malignancy, in agreement with the metabolic changes occurring in these cells [32].

The effect of natural mineral waters upon ^{14}C-BT or ^{3}H-OMG uptake are most probably dependent on their mineral content, as they were statistically different from the effect of distilled water alone. Thus, we also tested the effect of some minerals present in these waters. In relation to ^{14}C-BT uptake, our results suggest an inhibitory effect of Mg^{2+}, Ca^{2+}, Cl^-, or SO_4^{2-} upon it. We recently verified that ^{14}C-BT uptake in Caco-2 cells is Cl^- -dependent [46]; on the other hand, several *in vitro* and epidemiological studies describe a protective effect of either Ca^{2+} or Mg^{2+} against CCR [71–75], a negative correlation between Ca^{2+} and Mg^{2+} levels in drinking water and CCR being also described [76]. So, we conclude that Cl^- and possibly SO_4^{2-} are most probably responsible for the inhibitory effect of the minerals upon ^{14}C-BT uptake, and that other effects of Ca^{2+} or Mg^{2+} must be responsible for their protective role against CCR. These effects might include (a) binding of Ca^{2+} to biliary salts and fatty acids, causing an inhibition of biliary acids reabsorption and facilitating their excretion [77]; (b) the role of Mg^{2+} in stabilizing and repairing DNA [71, 78]; (c) the role of Ca^{2+} and Mg^{2+} in modulating cell proliferation and differentiation and the progression of the cell cycle [71, 75]; and (c) the role of Mg^{2+} as an anti-oxidant agent [79, 80].

In relation to the effect of ions upon the uptake of ^{3}H-OMG, our results suggest an inhibitory effect of both SO_4^{2-} and F^- upon this parameter, as we think that the inhibitory effect of $MgSO_4$ and NaF upon ^{3}H-OMG uptake is not related to the presence of Mg^{2+} or Na^+, respectively. In relation to Mg^{2+}, the lack of effect of $MgCl_2$ excludes the putative effect of Mg^{2+}. In relation to Na^+, a stimulatory effect of this ion on glucose intestinal absorption is very well documented [54, 55, 81, 82], and so a reduction in the uptake of ^{3}H-OMG by this ion is not unexpected???. In agreement with our results, F^- was found to reduce glucose uptake by isolated epithelial cells of rat intestine [83]. Moreover, bicarbonate-sulfur mineral waters are indicated in diabetes, because they decrease glycemia, polydipsia, and polyuria, and they reduce insulin requirements [1]. Based on our results, we hypothesize that a reduction of glucose intestinal absorption might contribute to the anti-diabetic effect of sulfur waters.

In conclusion, uptake of ^{14}C-BT and ^{3}H-OMG by Caco-2 cells is differently modulated by distinct natural mineral waters, either after acute or chronic exposure. It is well known that glucose intestinal absorption plays an important role in maintaining plasma glucose homeostasis, which is an important protective factor against diseases such as diabetes and obesity, and that BT plays an important role in maintaining colonic epithelial homeostasis. Even considering that caution must be maintained in extrapolating from *in vitro* studies to human diseases and health, and that the effect of mineral waters might be changed in the

presence of other substances (e.g., food), the results of this study seem, for the reasons mentioned above, interesting.

Acknowledgement

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In Vitro Studies on the Inhibition of Colon Cancer by Butyrate and Polyphenolic Compounds

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Our aim was to investigate the effect of several dietary polyphenols on uptake of ^{14}C-butyrate (^{14}C-BT) by Caco-2 cells and try to correlate this effect with the modulation of the anticarcinogenic effect of BT in these cells. Acutely, uptake of ^{14}C-BT (10 μM) was decreased by resveratrol, quercetin, myricetin, and chrysin, and increased by xanthohumol, catechin, and epicatechin; and uptake of ^{14}C-BT (20 mM) was reduced by resveratrol, quercetin, myricetin, chrysin, EGCG, and epicatechin. Resveratrol acts as a competitive inhibitor of ^{14}C-BT uptake. Chronically, quercetin and EGCG increased uptake of ^{14}C-BT (10 μM), whereas myricetin, rutin, chrysin, and xanthohumol decreased it. Moreover, catechin (1 μM), quercetin, myricetin, rutin, EGCG, and chrysin increased uptake of ^{14}C-BT (20 mM), whereas catechin (0.1 μM) decreased it. EGCG, myricetin, and catechin decreased MCT1 mRNA expression, while chrysin increased it; quercetin, rutin, and xanthohumol had no effect. BT (5 mM; 48 h) markedly decreased cellular viability and proliferation and increased cell differentiation and apoptosis. In general, combination of polyphenolic compounds with BT did not significantly modify these changes. In conclusion, changes in uptake of BT induced by polyphenols do not correlate with changes on the effect of BT upon cell viability, cell proliferation, differentiation, and apoptosis.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common solid tumors worldwide, being the second leading cause of cancer death in the United States. The causes of CRC are multifactorial, but an association between reduced risk of CRC and diets high in fruit, fiber, and vegetables has been well established in epidemiological studies (1,2).

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Naturally occurring plant polyphenols have recently come into scientific focus in terms of their potential chemopreventive role in CRC development because of their presence in various popular natural products (e.g., wine grapes, beer, teas, berries) and, more importantly, due to their reported anticarcinogenic effect in various animal studies and cell models, through influences in molecular events involved in initiation, promotion, and progression stages of several types of cancer, including CRC (3,4). Several proposed mechanisms explain the anticarcinogenic effect of polyphenols: antioxidant scavenging of free radicals; regulation of signal transduction pathways of cell growth and proliferation; suppression of oncogenes and tumor formation; anti-survival effect and induction of apoptosis; anti-inflammatory effect; inhibition of angiogenesis and metastasis; modulation of enzyme activity related to detoxification, oxidation, and reduction; stimulation of the immune system and DNA repair; and regulation of hormone metabolism (3,4).

Short-chain fatty acids (SCFA; acetate, propionate, and butyrate) are main end-products of anaerobic bacterial fermentation of dietary fiber within the human colon. Among SCFA, butyrate (BT) plays a key role in colonic epithelium homeostasis, having multiple important roles at that level: (1) is the main energy source for colonocytes (5,6); (2) inhibits colon carcinogenesis (7,8); (3) promotes growth and proliferation of normal colonic epithelial cells (9,10); (4) stimulates fluid and electrolyte absorption (11,12); (5) inhibits colon inflammation and oxidative stress; and (6) improves the colonic defense barrier function (13,14). As stated, one of the proposed beneficial effects of BT on human intestinal health is the prevention/inhibition of colon carcinogenesis. Most of the epidemiological studies showed an inverse relationship between dietary fiber intake and the incidence of CRC (1,2), and exposure of many colon tumor cell lines to BT leads to anticarcinogenic effects by induction of cell differentiation and apoptosis and by inhibition of proliferation

(7,8). At the molecular level, the anticarcinogenic effect of BT is considered to depend on regulation of gene expression, which is often attributed to its inhibition of histone deacetylase (15,16), resulting in hyperacetylation of histones and enhancement of the accessibility of transcription factors to nucleosomal DNA (17).

BT is transported into colonic epithelial cells by two specific carrier-mediated transport systems, MCT1 (18,19) and SMCT1 (20). In the context of colon carcinogenesis, it is very interesting to verify that both MCT1 (21) and SMCT1 (20) were recently proposed to function as tumor suppressors. So both SMCT- and MCT1-mediated transport of BT into colonocytes is fundamental for the regulation of cell homeostasis.

Interestingly enough, a number of reports have shown that some polyphenols can interplay with MCT1, suggesting a potential for polyphenols–BT interaction at this level (22–26). The aim of this study was to investigate the effect of several dietary polyphenols upon uptake of ^{14}C-BT by a human colonic adenocarcinoma cell line (Caco-cells) and try to correlate this effect with the modulation of the anticarcinogenic effect of BT in these cells.

MATERIALS AND METHODS

Caco-2 Cell Culture

The Caco-2 cell line was obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage number 31–78. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm; Corning Costar, Corning, NY).

Determination of ^{14}C-BT Uptake by Caco-2 Cells

For uptake studies, Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; Ø 16 mm; Corning Costar), and the experiments were performed 8–11 days after the initial seeding. The cell medium was made free of fetal calf serum for 24 h before the experiments. The uptake experiments were performed with Caco-2 cells incubated in glucose-free Krebs buffer (containing, in mM: 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 NaHCO3, 1.6 KH_2PO_4, 0.4 K_2HPO_4, and 20 MES (pH 6.5)). Initially, the culture medium was aspirated and the cells were washed with 0.3 ml buffer at 37°C. Then the cell monolayers were preincubated for 20 min in 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.3 ml medium at 37°C containing either 10 μM or 20 mM ^{14}C-BT (except in the experiments for determination of kinetics of uptake). Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.5 ml ice-cold buffer. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris.HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Acute Effect of Compounds

The acute effect of compounds on ^{14}C-BT uptake was tested by preincubating and incubating cells with ^{14}C-BT in the presence of the compounds to be tested.

Chronic Effect of Compounds

The chronic effect of compounds on ^{14}C-BT uptake was tested by cultivating 6–9-day-old cell cultures (90–95% confluence) in culture medium in the presence of the compounds to be tested. The medium was renewed daily, and the transport experiments were performed after 48 h. The transport experiments were identical to the experiments described above, except that there was no preincubation period, and cells were incubated with ^{14}C-BT in the absence of drugs.

Effect of Compounds on Cell Viability [MTT (3-{4,5-Dimethyl-2-Thiazolyl}-2,5-Diphenyl-2H-Tetrazolium Bromide) Assay]

To test whether the compounds that had an acute effect upon ^{14}C-BT uptake affected cellular viability, cells were incubated for 3 h at 37°C in 500 μl of culture medium with MTT solution (0.5 mg/ml). In the last 23 min of this period, the compounds to be tested were added. To test whether the compounds that had a chronic effect upon ^{14}C-BT uptake affected cellular viability, cells were chronically treated with the compounds as described above. After 45 h of treatment, 50 μl MTT solution (5 mg/ml) was added. The cells were then further incubated for 3 h at 37°C. The MTT solution was removed after the 3-h incubation period, and the cells were lysed by addition of 200 μl DMSO followed by plate shaking for 10 min at room temperature. Optical density (OD) for the solutions in each well was determined at both 550 nm and 650 nm. OD at 650 nm corresponds to unspecific light absorption and was subtracted from the OD at 550 nm to give the OD value specific to formazan crystals derived from MTT cleavage (27).

Quantitative Reverse Transcription Real-Time PCR

Total RNA was extracted from chronically treated Caco-2 cells using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Germany).

Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions, and 0.5 μg of resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 20 μl of final reaction volume, according to the manufacturer's

instructions. The resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade unreacted RNA. For the quantitative real-time PCR, 2 μl of the 20 μl reverse transcription reaction mixture was used. For the calibration curve, Caco-2 standard cDNA was diluted in 5 different concentrations.

The primer pairs used for amplification/quantification were 5′-CAC CGT ACA GCA ACT ATA CG-3′ (forward) and 5′-CAA TGG TCG CCT CTT GTA GA-3′ (reverse) for MCT1 (34) and 5′-ATG GAG AAG GCT GGG GCT CAT-3′ (forward) and 5′-GAC GAA CAT GGG GGC ATC AG -3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ). Twenty μl reactions were set up in microcapillary tubes using 0.5 μM of each primer and 4 μl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 10 s, and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment] repeated 45 and 37 times (for MCT1 and GAPDH, respectively), a melting curve program [(AT+10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement], and a cooling step to 40°C (30 s). The annealing temperatures were 60°C and 65°C, for MCT1 and GAPDH genes, respectively. Data were analyzed using LightCycler analysis software (Roche, Mannheim, Germany).

Determination of ^{14}C-BT Oxidation

To evaluate the chronic effect of the compounds on the oxidation of BT, we determined ^{14}C-BT usage as an energy source in Caco-2 cells. For that, production of $^{14}CO_2$ resulting from the metabolism of ^{14}C-BT was determined by a method adapted from (28,29). Basically, Caco-2 cells were seeded in 9.2 cm^2 plates (TPP), and after 5 days of culture, cells were cultured in medium containing compounds to be tested or the respective solvents for 48 h (with medium change after 24 h). At the end of that period, cells were incubated for 90 min with 600 μl of culture medium containing ^{14}C-BT (10 μM). After that, a paper filter humidified with 600 μl of NaOH 1M (which absorbs CO_2 from the vapor phase) was placed in the lid of the plate, and 55 μl of 1.5 M citric acid, pH 5.0, was added to the culture medium (it stops cell metabolism and volatiles the $^{14}CO_2$). The plates were then sealed with Parafilm and incubated for 3 h at 4°C. The radioactivity in the filter was then measured by liquid scintillation counting. Results are expressed in nmol $^{14}CO_2$/mg/protein.

Determination of Cell Viability

Caco-2 cells were seeded in 24-well plates (TPP), and after 5 days of culture, cells were cultured in medium containing compounds to be tested or the respective solvents for 48 h (with medium change after 24 h). Two different methods were used to determine cell viability:

Trypan Blue Extrusion

Membrane integrity was determined microscopically by trypan blue (0.4%) dye exclusion assay.

Quantification of Extracellular LDH Activity

Cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular medium was measured spectrophotometrically by measuring the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described by Negrão et al. (30).

Determination of Cellular Proliferation (Quantification of Methyl-^{3}H-Thymidine Incorporation)

Quantification of cellular DNA synthesis rate was obtained by measuring the incorporation of methyl-^{3}H-thymidine into cellular DNA. For this, Caco-2 cells were seeded in 24-well plates (TPP), and after 5 days of culture, cells were cultured in medium containing compounds to be tested or the respective solvents for 48 h (with medium change after 24 h). Then, 500 μl of culture medium containing methyl-^{3}H-thymidine (0.125 μCi/ml) was added for 5 h. After this period, the medium was removed and the cells were fixed by incubation with 300 μl 10% trichloroacetic acid (TCA) (1 h at 4°C). Then, the cells were washed twice with 10% TCA to remove unbound radioactivity, plates were air-dried for 30 min, and, finally, the cells were lysed with 1 M NaOH (280 μl/well). A 250-μl aliquot of the lysate was neutralized with HCl prior to the addition of scintillation fluid. The radioactivity of the samples was quantified by liquid scintillation counting. Cellular DNA synthesis rate was expressed as incorporation of methyl-^{3}H-thymidine/well.

Determination of Cell Differentiation (Alkaline Phosphatase Activity Assay)

Caco-2 cells were seeded in 24-well plates (TPP), and after 5 days of culture, cells were cultured in medium containing compounds to be tested or the respective solvents for 48 h (with medium change after 24 h). Cell differentiation was measured by alkaline phosphatase (ALP) activity, as previously described (31). ALP activity was determined spectrophotometrically, using *p*-nitrophenylphosphate as substrate, and the results are expressed as nmol *p*-nitrophenol/min/mg protein.

Determination of Apoptosis Index (TUNEL Assay)

One-day-old Caco-2 cell cultures were cultivated in medium containing compounds to be tested or the respective solvents for 48 h (with medium change after 24 h). Then, the apoptosis index was determined by the TUNEL assay, according to the instructions of the producer (TUNEL kit; In Situ Cell Death Detection, Roche Diagnostics, Basel, Switzerland). Briefly, cells were fixed with 4% *p*-formaldehyde solution in PBS for 30 min, permeabilized [with sodium citrate 0.1% (w/v) and triton X-100 0.1% (v/v)] for 2 min at 4°C, and then incubated with fluorescein isothiocyanate-conjugated dUTP for 1 h at 37°C. DAPI (0.5 μg/ml in methanol, 5 min) was used to stain total

nuclei. Coverslips were mounted on glass slides and visualized under a fluorescence microscope (Nikon 50i, Nikon, Japan). Fluorescein-labeled and DAPI-labeled nucleis were counted in 7 randomly chosen optical fields per slide (in a total of about 10,000 nuclei), and apoptotic cells presented as a percentage of total cells.

Protein Determination

The protein content of cell monolayers was determined as described by Bradford et al. (32), using human serum albumin as standard.

Calculation and Statistics

For the analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis-Menten equation were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method (33).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. Statistical significance of the difference between two groups was evaluated by Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance (ANOVA) test, followed by the Bonferroni test. Differences were considered to be significant when $P < 0.05$.

Materials

Materials used were ^{14}C-BT [*n*-butyric acid, sodium salt (1-^{14}C); specific activity 30–60 mCi/mmol; Biotrend Chemikalien GmbH, Köln, Germany]; (+)catechin hydrate, chrysin, epicatechin, EGCG ((−) epigallocatechin-3-gallate]), MES [2-(N-morpholino)ethanesulfonic acid hydrate], myricetin, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), quercetin dihydrate, resveratrol, rutin, trypsin-EDTA solution (Sigma, St. Louis, MO); DMSO (dimethylsulfoxide), Triton X-100 (Merck, Darmstadt, Germany); and fetal calf serum (Invitrogen Corporation, Carlsbad, CA).

Xanthohumol was kindly donated by Eng. José M. Machado Cruz, from iBeSa—Instituto de Bebidas e Saúde (S. Mamede Infesta, Portugal). Drugs to be tested were dissolved in ethanol or DMSO, the final concentration of these solvents being 1% in the buffer or 0.1% in the culture media for acute or chronic treatments, respectively. Controls for these drugs were run in the presence of the solvent.

RESULTS

Previously, our group have shown that ^{14}C-BT apical uptake in Caco-2 cells was found to be linear with time for up to 3 min of incubation (34). So, in this work, cells were incubated with ^{14}C-BT for 3 min, in order to determine initial rates of uptake.

Acute Effect of Polyphenols Upon the Apical Uptake of ^{14}C-BT

The acute effect of several different polyphenolic compounds was investigated (Fig. 1A). Of these, resveratrol, quercetin, myricetin, and chrysin were found to reduce ^{14}C-BT (10 μM) uptake. For most of the compounds, the inhibitory effect was not very pronounced, but resveratrol (100 μM) was able to cause a 30% reduction in ^{14}C-BT uptake. On the other hand, xanthohumol, catechin, and epicatechin caused small but significant increases in ^{14}C-BT uptake and rutin and EGCG were devoid of effect (Fig. 1A).

The polyphenolic compounds were also tested against a high concentration of ^{14}C-BT (20 mM). Resveratrol, quercetin, myricetin, chrysin, EGCG, and epicatechin, at least in some of the concentrations tested, were able to significantly reduce uptake of ^{14}C-BT by a maximum of 10–20%. Xanthohumol, rutin, and catechin showed no effect (Fig. 1B).

Acutely, viability was not affected by myricetin (100 μM), epicatechin (1 μM), and catechin (1 μM). However, it decreased in the presence of resveratrol (100 μM) and quercetin (100 μM) (to 86 ± 2 and 94 ± 3% of control, respectively; $n = 6$), and increased in the presence of rutin (100 μM), xanthohumol (10 μM), EGCG (10 μM), and chrysin (to 109 ± 2, 109 ± 1, 109 ± 3, and 112 ± 3% of control, respectively; $n = 6$).

Chronic Effect of Polyphenols Upon the Apical Uptake of ^{14}C-BT

The same polyphenolic compounds were tested over a 48-h period. As shown in Fig. 2A, quercetin and EGCG increased uptake of ^{14}C-BT (10 μM) by Caco-2 cells (by 22 and 14%, respectively), while myricetin, rutin, chrysin, and xanthohumol decreased it (by 8%, 13%, 18%, and 11%, respectively). In contrast, resveratrol, catechin, and epicatechin were devoid of effect (Fig. 2A).

Moreover, as shown in Fig. 2B, quercetin, myricetin, rutin, EGCG, chrysin, and catechin (1 μM) increased uptake of ^{14}C-BT (20 mM) by Caco-2 cells (by a maximum of 11%, 21%, 31%, 54%, 58%, and 32%, respectively). In contrast, catechin (0.1 μM) decreased it (by 11%). Finally, resveratrol, xanthohumol, and epicatechin were devoid of effect (Fig. 2B).

Chronically, most of the polyphenols tested were devoid of significant effect upon cell viability [namely, resveratrol, quercetin, epicatechin (10 μM), myricetin (1 μM), catequin (0.1 μM), and chrysin and rutin (1 and 10 μM)]. Nonetheless, viability was increased by EGCG (10 μM), catechin (1 μM), and xanthohumol (10 μM) (to 103 ± 1, 104 ± 1, and 136 ± 3% of control; $n = 4$–7), and decreased by myricetin (10 μM) (to 90 ± 2% of control; $n = 6$).

Effect of Resveratrol Upon the Kinetic Parameters of ^{14}C-BT Apical Uptake

Of the polyphenols tested, resveratrol showed the most marked effect, when tested acutely, upon ^{14}C-BT uptake. So we decided to test its effect upon the kinetic parameters of ^{14}C-BT apical uptake (Fig. 3). Resveratrol behaves as a competitive inhibitor of ^{14}C-BT apical uptake, as this compound significantly increased the K_m of uptake (from 3.16 ± 0.78 to 5.85 ± 0.79 mM; $P < 0.05$), without changing the V_{max} (this parameter

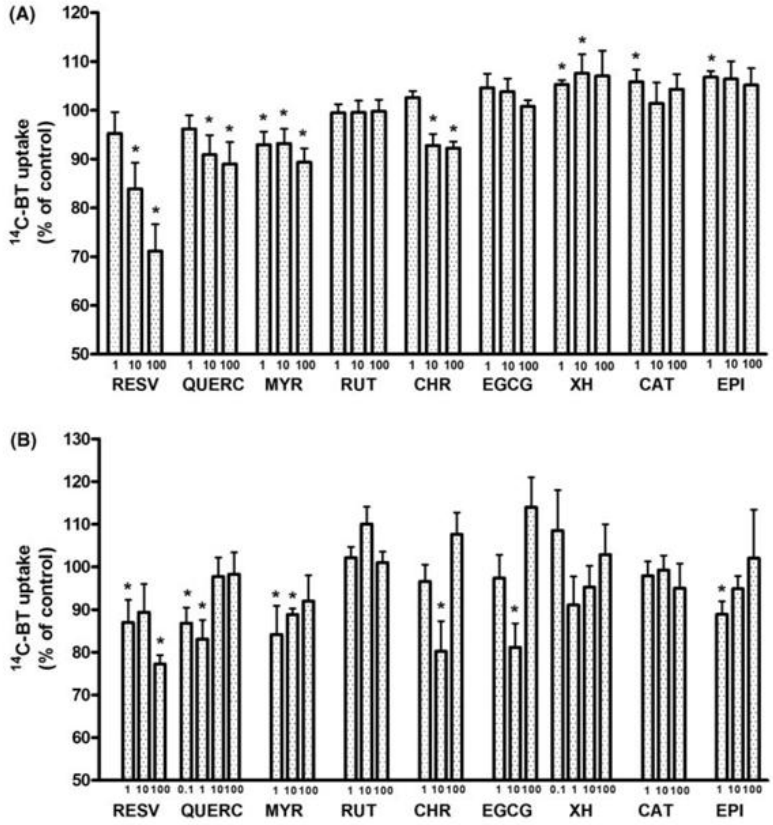

FIG. 1. Acute effect of polyphenols upon uptake of A: ^{14}C-BT 10 μM and B: ^{14}C-BT 20 mM. Initial rates of uptake were determined in Caco-2 cells preincubated (20 min) and then incubated (3 min) with ^{14}C-BT in the absence (control) or in the presence of resveratrol (RESV 1–100 μM; $n = 6$–9), quercetin (QUERC 1–100 μM; $n = 6$–9), myricetin (MYR 1–100 μM; $n = 9$), rutin (RUT 1–100 μM; $n = 5$–9), chrysin (CHR 1–100 μM; $n = 6$–9), EGCG (1–100 μM; $n = 6$–9), xanthohumol (XH 1–100 μM; $n = 6$–12), catechin (CAT 1–100 μM; $n = 6$–9), or epicatechin (EPI 1–100 μM; $n = 6$–9). Shown are arithmetic means ± SEM. *Significantly different from control.

was found to be 70.3 ± 10.3 and 73.4 ± 7.1 nmol mg prot^{-1} 3 min^{-1} in the absence and presence of resveratrol, respectively).

Effect of Polyphenols Upon MCT1 mRNA levels

We next investigated the possibility of interference with mRNA expression levels of MCT1 as an explanation to the effect of polyphenolic compounds upon ^{14}C-BT uptake by Caco-2 cells. As shown in Fig. 4, some of the compounds tested significantly affected MCT1 expression. Indeed, EGCG (10 μM), myricetin (10 μM), and catechin (0.1 and 1 μM) caused significant decreases in MCT1 mRNA expression [of 26, 38, and 44% (maximum), respectively]. On the other hand, chrysin (1 and 10 μM) concentration-dependently increased MCT1 mRNA levels (to a maximum of 159% of control). Finally, the mRNA expression level of MCT1 was not affected by quercetin, rutin, and xanthohumol (Fig. 4).

Effect of Butyrate and of the Polyphenols Quercetin, EGCG, Rutin, and Chrysin Upon (a) ^{14}C-BT Oxidation and (b) Cell Viability, Proliferation, Differentiation, and Apoptosis

On the basis of the chronic (48 h) effects of the polyphenolic compounds upon uptake of ^{14}C-BT 10 μM and 20 mM (Fig. 2), we selected 4 compounds (quercetin, EGCG, rutin, and chrysin) for further experiments. In these experiments, their effect upon ^{14}C-BT oxidation, upon cell proliferation, viability, differentiation, and apoptosis, and upon changes in these parameters induced by BT were assessed.

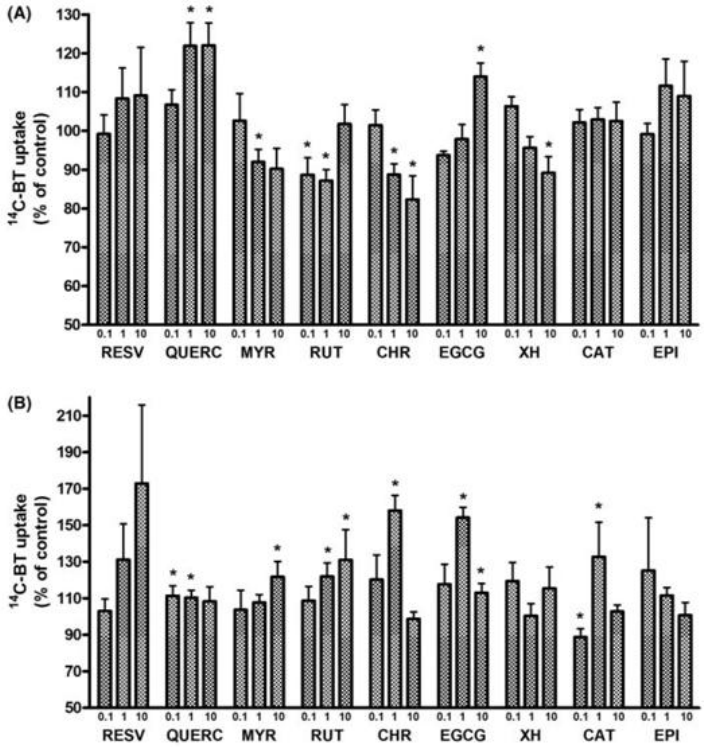

FIG. 2. Chronic effect of polyphenols upon uptake of A: ^{14}C-BT 10 μM and B: ^{14}C-BT 20 mM. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with 10 μM ^{14}C-BT, after a 48-h cell culture in the absence (control) or in the presence of resveratrol (RESV 0.1–10 μM; $n = 6$–9), quercetin (QUERC 0.1–10 μM; $n = 6$–9), myricetin (MYR 0.1–10 μM; $n = 6$–9), rutin (RUT 0.1–10 μM; $n = 6$–12), chrysin (CHR 0.1–10 μM; $n = 6$–9), EGCG (0.1–10 μM; $n = 6$–9), xanthohumol (XH 0.1–10 μM; $n = 6$–9), catechin (CAT 0.1–10 μM; $n = 6$–12), or epicatechin (EPI 0.1–10 μM; $n = 6$–9). Shown are arithmetic means ± SEM. *Significantly different from control.

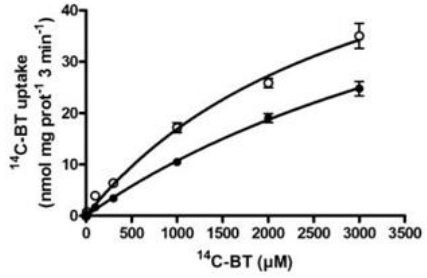

FIG. 3. Effect of resveratrol upon the kinetic parameters of the apical uptake of ^{14}C-BT by Caco-2 cells. Initial rates of uptake were determined in Caco-2 cells incubated with ^{14}C-BT for 3 min, in the absence (open circles) or in the presence of resveratrol 300 μM (closed circles) ($n = 8$). Shown are arithmetic means ± SEM.

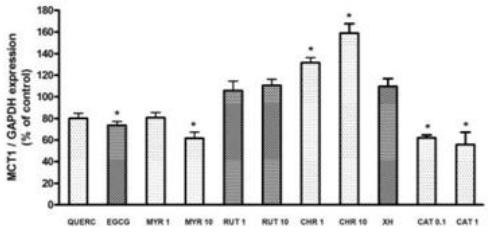

FIG. 4. Effect of polyphenols on the expression levels of MCT1 mRNA, as determined by qRT-PCR. Cells were treated for 48 h with quercetin 10 μM (QUERC), EGCG 10 μM (EGCG), myricetin 1 or 10 μM (MYR 1 and 10), rutin 1 or 10 μM (RUT 1 and 10), chrysin 1 or 10 μM (CHR 1 and 10), xanthohumol 10 μM (XH) or catechin 0.1 or 1 μM (CAT 0.1 and 1). Shown are arithmetic means ± SEM of the expression of each test gene relative to GAPDH ($n = 4$–5). *Significantly different from control.

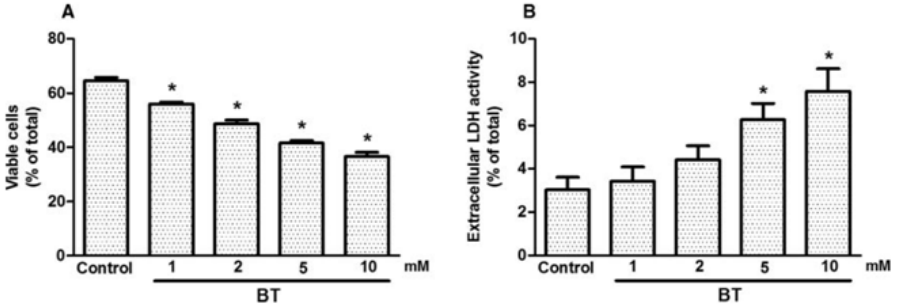

FIG. 5. Effect of chronic exposure (48 h) to increasing concentrations of butyrate (BT) (1, 2, 5, or 10 mM) on Caco-2 cell viability, as determined by A: the trypan blue extrusion method ($n = 8–9$), and B: quantification of extracellular lactate dehydrogenase activity ($n = 6–8$). Results are shown as arithmetic means + SEM. *Significantly different from control.

In preliminary experiments, the effect of chronic (48 h) treatment of the cells with increasing concentrations of BT (1, 2, 5, and 10 mM) upon cell viability was assessed with two different methods (Fig. 5). On the basis of the results obtained, we selected BT 5 mM for further experiments.

Effect Upon ^{14}C-BT Oxidation

In this series of experiments, the effect of BT and polyphenols upon oxidation of ^{14}C-BT by Caco-2 cells was investigated. Treatment of the cells for 48 h with BT (5 mM) produced a 20% decrease in ^{14}C-BT oxidation (Fig. 6). Quercetin, EGCG, and chrysin, but not rutin, also decreased ^{14}C-BT oxidation (by 13–15%). However, combination of BT with the polyphenols did not significantly change the effect of BT alone upon ^{14}C-BT oxidation, although some tendency for a higher decrease in this parameter was observed (Fig. 6).

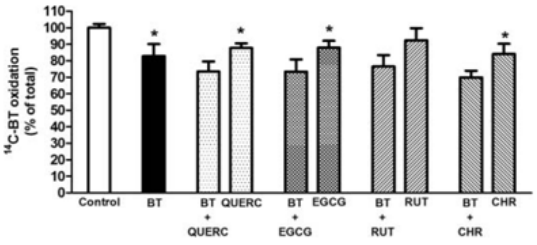

FIG. 6. Effect of chronic exposure (48 h) to butyrate 5 mM (BT), to BT+quercetin (BT+QUERC), EGCG (BT+EGCG), rutin (BT+RUT), or chrysin (BT+CHR) (10 μM) or to QUERC, EGCG, RUT, or CHR (10 μM) upon oxidation of ^{14}C-BT by Caco-2 cells. Results are shown as CPM/mg protein (% of control) (arithmetic means + SEM; $n = 7–9$). *Significantly different from control, #significantly different from BT.

Effect Upon Cell Viability, Proliferation, Differentiation, and Apoptosis

The effects of BT alone or combined with polyphenols on Caco-2 cell viability were examined after 48 h of treatment. As shown in Fig. 7, BT (5 mM) alone caused a marked (2–3-fold) increase in cell death. Quercetin, EGCG, rutin, and chrysin alone (0.1–10 μM) did not have a significant effect on cell viability. Moreover, they also did not change the increase in cell death produced by BT (Fig. 7).

In the next series of experiments, the effects of BT and polyphenols on Caco-2 cell proliferation were analyzed. Treatment of the cells for 48 h with BT (5 mM) induced a significant (25–30%) decrease in cell proliferation (Fig. 8). Rutin had no effect upon proliferation; curiously, low concentrations of quercetin, EGCG, and chrysin slightly decreased proliferation, but higher concentrations of these compounds caused a small but significant increase in Caco-2 proliferation (Fig. 8). Moreover, combination of BT with polyphenols did not change the inhibitory effect of BT; the only two exceptions were found with rutin (0.1 μM) and chrysin (10 μM). In the presence of these compounds, a small but significant enhancement and decrease, respectively, of the inhibitory effect of BT upon proliferation was found (Fig. 8).

Next, the effect of BT and polyphenols upon cell differentiation was studied. BT alone caused a significant increase in cell differentiation, as shown by the 50% increase in ALP activity. Most of the polyphenols tested did not change this parameter; however, rutin (at all concentrations tested) decreased differentiation. Combination of BT with polyphenols did not change the effect of BT upon cell differentiation (Fig. 9).

In the last series of experiments, we aimed at investigating the effect of BT and polyphenols upon Caco-2 cells apoptosis. As shown in Fig. 10, BT (5 mM) produced a very marked (17×) increase in apoptosis index after 48 h. Quercetin, EGCG, rutin, and chrysin (10 μM) alone also induced a significant increase in apoptosis (6–10×). The combination of BT and the polyphenols

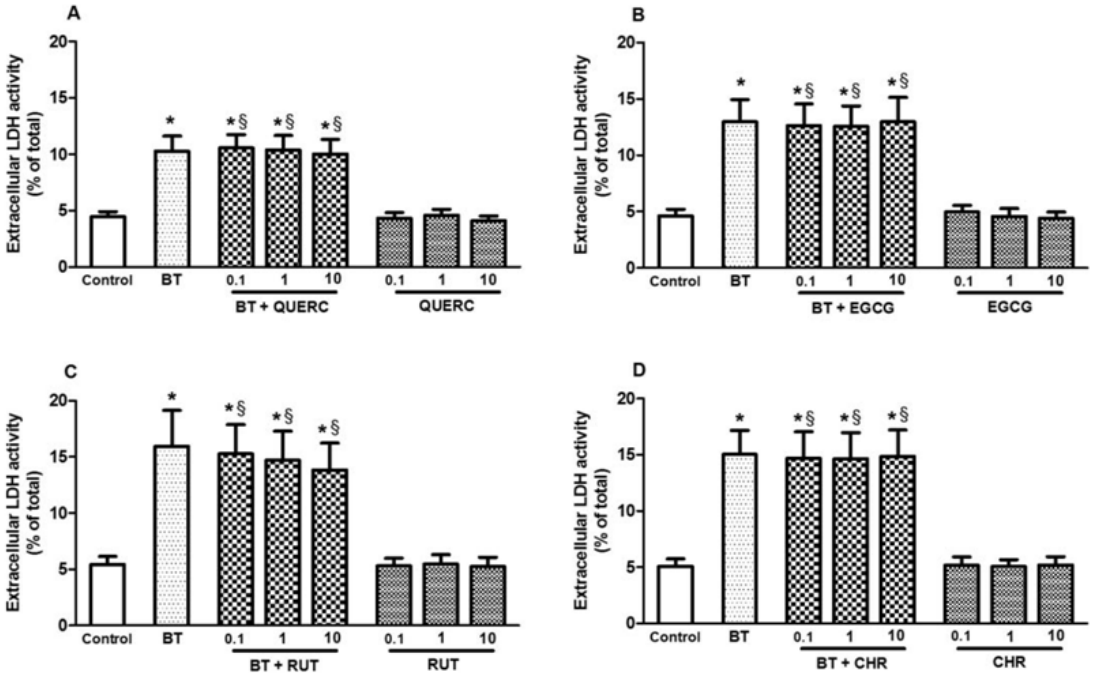

FIG. 7. Effect of chronic exposure (48 h) to butyrate 5 mM (BT), to BT + polyphenolic compound (0.1, 1, or 10 μM) or to polyphenolic compound (0.1, 1 or 10 μM) on Caco-2 viability, determined by quantification of extracellular lactate dehydrogenase (LDH) activity. A: quercetin (QUERC); B: EGCG; C: rutin (RUT); and D: chrysin (CHR). Results are shown as extracellular LDH activity (% of total activity) (arithmetic means + SEM; $n = 12$). *Significantly different from control, #significantly different from BT, §significantly different from the polyphenolic compound (0.1, 1, or 10 μM).

induced also a significant apoptotic effect. However, this effect was not greater than that observed with BT alone (Fig. 10).

DISCUSSION

Because BT plays an essential role in the maintenance of colonic tissue homeostasis, an understanding of the regulation of its absorption by the colon mucosa appears particularly important (35). However, very little is known concerning this subject. It is known that MCT1-mediated intestinal epithelial absorption of BT is upregulated by its substrate, BT (36), enhanced by leptin (37), phorbol 12-myristate 13-acetate (38), caffeine, and acetylsalicylic acid (34), and inhibited by enteropathogenic *E. coli* (39), interferon-γ, tumor necrosis factor-α (28), theophylline, tetrahydrocannabinol, MDMA (ecstasy), acetaldehyde, and indomethacin (34). Interestingly enough, some dietary polyphenolic compounds were also found to interfere with MCT1 (22–26). Because polyphenolic compounds have reported anticarcinogenic effect in various in vitro and in vivo models (40,41), we decided to further investigate the relationship between these compounds and BT uptake by Caco-2 cells, which was previously found to be MCT1-mediated (34). Furthermore, we tried to correlate their effect upon BT uptake with the modulation of the anticarcinogenic effect of BT in these cells.

First, we investigated the effect of several polyphenols upon ^{14}C-BT uptake. The compounds to investigate (resveratrol, quercetin, myricetin, rutin, chrysin, EGCG, xanthohumol, catechin, and epicatechin) as well as the concentrations to test (both acute and chronically) were selected based on previous works from our group showing that they interfere with the membrane uptake of several distinct compounds in different cell types (42–45). The effects of the compounds was analyzed both at a low (10 μM) and at a high (20 mM) concentration of ^{14}C-BT. These two concentrations have physiological relevance. On the one hand, a high concentration (in the mM range) of BT in the colonic lumen may be attained, for example, after digestion of dietary fiber (the concentrations of these fatty acids in the lumen may reach 70–130 mM, with a 20–30% BT). On the other hand, a low concentration (in the μM range) of BT may be attained, for example, in the intermeal period, at the bottom of colonic crypts or in the inner layers of tumors (34).

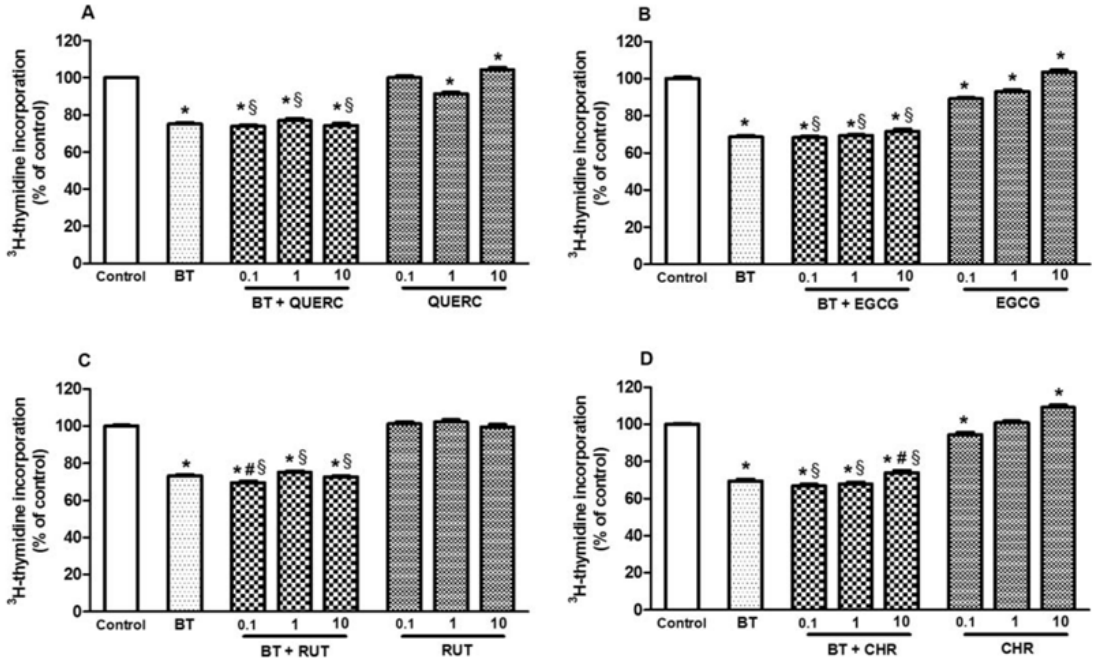

FIG. 8. Effect of chronic exposure (48 h) to butyrate 5 mM (BT), to BT + polyphenolic compound (0.1, 1 or 10 μM), or to polyphenolic compound (0.1, 1, or 10 μM) on Caco-2 cellular proliferation, determined by quantification of ^{3}H-thymidine incorporation. A: quercetin (QUERC); B: EGCG; C: rutin (RUT); and D: chrysin (CHR). Results are shown as μCi/well (% of control) (arithmetic means + SEM; $n = 8$–9). *Significantly different from control, #significantly different from BT, §significantly different from the polyphenolic compound (0.1, 1, or 10 μM).

The results of the present study clearly demonstrate that uptake of ^{14}C-BT by Caco-2 cells is modulated by several distinct polyphenolic compounds. Acutely, uptake of a low concentration of ^{14}C-BT (10 μM) was decreased by resveratrol, quercetin, myricetin, and chrysin, and increased by xanthohumol, catechin, and epicatechin. Uptake of a high concentration of ^{14}C-BT (20 mM) was reduced by resveratrol, quercetin, myricetin, chrysin, EGCG, and epicatechin. Resveratrol behaved as a competitive inhibitor of ^{14}C-BT uptake, as it increased the K_m of transport without changing the V_{max}. Chronically, quercetin and EGCG increased uptake of ^{14}C-BT (10 μM), whereas myricetin, rutin, chrysin, and xanthohumol decreased it. Moreover, catechin (1 μM), quercetin, myricetin, rutin, EGCG, and chrysin increased uptake of ^{14}C-BT (20 mM), whereas catechin (0.1 μM) decreased it. For some of the compounds (catequin 0.1 μM and chrysin 1 μM), changes in ^{14}C-BT uptake correlated well with changes in MCT1 mRNA expression levels, suggesting that their effect upon ^{14}C-BT uptake results from changes in MCT1 transcription levels. For all the other compounds, however, such a correlation was not found. This discrepancy suggests that the effect of these polyphenols upon ^{14}C-BT uptake is not the result of a change in the transcription levels of MCT1 but is probably related to changes in the protein levels or in the intrinsic activity of this transporter. Because polyphenolic compounds are known to affect cellular signal transduction pathways (3,4), the second hypothesis seems more attractive.

Concerning the effect of polyphenols upon ^{14}C-BT uptake, three points should be noted: (1) the inhibitory or stimulatory effect of the compounds upon the apical uptake of ^{14}C-BT was not related to an effect upon cellular viability. Indeed, with some exceptions [resveratrol (100 μM) and quercetin (100 μM) (acute) and xanthohumol (10 μM), EGCG (10 μM) and catechin (1 μM) (chronic)], no effect of the compounds upon cell viability was observed or no correlation between their effect upon ^{14}C-BT uptake and cell viability and found; (2) with some exceptions, the acute and chronic effects of polyphenols upon the apical uptake of ^{14}C-BT were distinct, and so care should be taken when extrapolating about chronic effects from acute effects and vice versa; and (3) with some exceptions, the effect of polyphenols upon the apical uptake of a low and a high concentration of ^{14}C-BT was also distinct. This point is very interesting in the context of the known variation in the intraluminal concentration of BT that exists between, for example, the fasting and the feeding period.

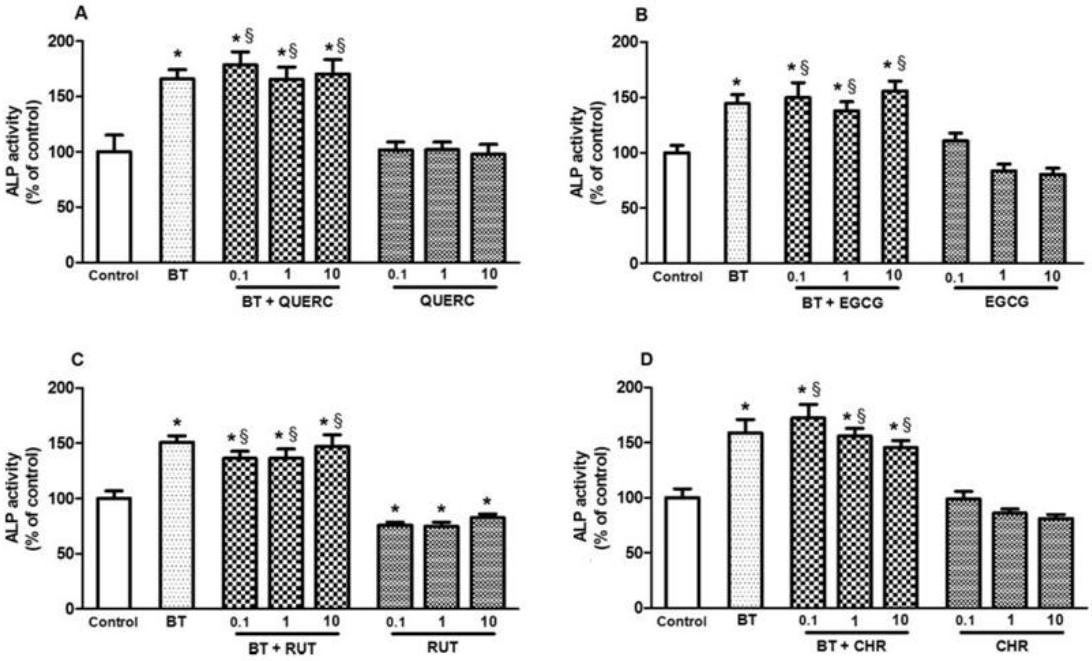

FIG. 9. Effect of chronic exposure (48 h) to butyrate 5 mM (BT), to BT + polyphenolic compound (0.1, 1, or 10 μM), or to polyphenolic compound (0.1, 1, or 10 μM) on Caco-2 cell differentiation, determined by quantification of alkaline phosphatase (ALP) activity. A: quercetin (QUERC); B: EGCG; C: rutin (RUT); and D: chrysin (CHR). Results are shown as nmol *p*-nitrophenol/min/mg protein (% of control) (arithmetic means + SEM; $n = 12$–15). *Significantly different from control, #significantly different from BT, §significantly different from the polyphenolic compound (0.1,1, or 10 μM).

Previously, the green tea flavonoids EGCG and epicatechin-3-gallate were found to be potent inhibitors of MCT1-mediated uptake in Caco-2 cells (23,25), and, although less markedly, catechin and epicatechin were also found to be MCT1 inhibitors (25). Also, the flavonoid quercetin and its metabolites naringenin, morin, and silybin were also shown to be MCT1 inhibitors in Caco-2 cells (24). Moreover, in other cell types, MCT1-mediated transport was inhibited by several flavonoids (apigenin, biochanin A, chrysin, diosemin, fisetin, genistein, hesperitin, kaempferol, luteolin, morin, narigenin, phloretin, and quercetin), but not by others (phloridzin and rutin) (22,26). In the present study, we extend the previous findings obtained with some flavonoids to another class of polyphenols, the stilbenes. Moreover, we verified that some polyphenolic compounds caused an increase in the uptake of ^{14}C-BT by Caco-2 cells. This observation is very interesting, as MCT1 and SMCT1 were recently proposed to function as tumor suppressors, a decrease in the expression/activity of these transporters being observed during transition from normality to malignancy in the colon (20,21). So an increase in the activity of MCT1 might be involved in the anticarcinogenic effect of at least some of the polyphenolic compounds.

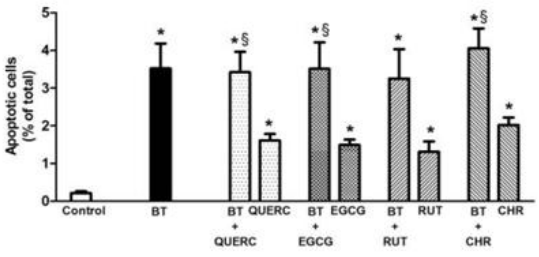

FIG. 10. Effect of chronic exposure (48 h) to butyrate 5 mM (BT), to BT + quercetin (QUERC), EGCG, rutin (RUT), or chrysin (CHR) (10 μM), or to QUERC, EGCG, RUT, or CHR (10 μM) on Caco-2 apoptosis index, determined by the TUNEL assay. Results are shown as % of apoptotic cells (arithmetic means + SEM; $n = 4$). *Significantly different from control, #significantly different from BT, §significantly different from the polyphenolic compound.

Four of the polyphenolic compounds (quercetin, EGCG, rutin, and chrysin) were able to significantly increase ^{14}C-BT uptake when chronically tested. Because of the aforementioned relationship between anticarcinogenesis and MCT1 activity, we decided to evaluate the effect of these four flavonoids on

cell viability, proliferation, differentiation, and apoptosis. We verified that the four polyphenolic compounds tested (EGCG, quercetin, rutin, and chrysin) (0.1–10 μM; 48 h) had no effect on Caco-2 cell viability. However, quercetin, EGCG, and chrysin decreased cell proliferation at low concentrations while increasing it at higher concentrations. Moreover, rutin decreased cell differentiation and the four compounds markedly increased apoptosis.

Our results obtained with EGCG and quercetin are in agreement with previous descriptions of an in vitro antiproliferative and proapoptotic effect of EGCG (46–49) and quercetin (50,51) in colon cancer cells, and of an in vivo inhibition of rat colorectal carcinogenesis by quercetin (52–55). As for rutin, nothing was known concerning its effects upon colon cancer cells. Our study, showing that rutin decreased cell differentiation and increased apoptosis in Caco-2 cells, is thus the first describing in vitro effects of rutin in colon cancer cell lines that might explain its anticarcinogenic effect found against CRC in vivo (52,54,56,57). As for chrysin, our results confirm the previous antiproliferative effect described in colon cancer cells (58) and further describe a proapoptotic effect of this compound; taken together, these effects might explain the anticarcinogenic effect of this compound against CRC in vivo (59). An important point must, however, be addressed: Although quercetin, EGCG, and chrysin showed antiproliferative effects at low concentrations, they presented a pro-proliferative effect when tested in higher concentrations. This raises the hypothesis that there is a fine line between anticarcinogenic/procarcinogenic effects of these compounds. This may be related to the fact that, although these compounds are described as antioxidants, the balance between their antioxidant and prooxidant activity is concentration-dependent (60).

Next, we also evaluated whether these polyphenols were able to modulate the anticarcinogenic effect of BT. In agreement with previous works (61,62), BT (5 mM; 48 h) was found to exert potent anticarcinogenic effects on Caco-2 cells by markedly decreasing cellular viability and proliferation while increasing cell differentiation and apoptosis. However, in general, combination of polyphenolic compounds with BT did not significantly modify the changes in proliferation, differentiation, cell death, and apoptosis induced by BT alone. Nevertheless, the combination of BT with polyphenols exhibits a greater modulation of almost all the parameters, relative to that produced by individual polyphenolic compounds. This suggests an enhanced chemopreventive efficacy of polyphenols when associated with BT. Knowing that polyphenols and dietary fiber are simultaneously present in fruits and vegetables, this observation seems very interesting.

The lack of modulation of BT effects by polyphenols might have several explanations, but one that seems very attractive is related to the fact that chronic polyphenols are known to increase the expression of efflux transporters such as MDR1 and MRP2 in Caco-2 cells (63,64). So, besides increasing BT uptake by the cells, polyphenols may also have caused an increase in the efflux of BT, thus having no significant effect on its intracellular concentrations. Because with our experimental conditions (3-min ^{14}C-BT incubation) we were measuring the effect of polyphenols on ^{14}C-BT uptake only, we could not observe their effect on efflux. This hypothesis would also explain the lack of effect of polyphenols on ^{14}C-BT oxidation when associated with BT (see below).

The effect of BT and of quercetin, EGCG, rutin, and chrysin upon ^{14}C-BT oxidation by Caco-2 cells was also evaluated. Chronic (48 h) exposure of the cells to BT, quercetin, EGCG, and chrysin caused a decrease in ^{14}C-BT oxidation by the cells, but the combination of BT and the polyphenolic compounds did not further decrease ^{14}C-BT oxidation. Interestingly enough, BT is known to upregulate MCT1 and thus increases its activity (36), and the polyphenolic compounds were also found, in the present work, to increase MCT1-mediated transport. So an increase in MCT1-mediated transport (originating an increase in the intracellular concentration of ^{14}C-BT) seems to originate, by a yet unknown mechanism, a decrease in ^{14}C-BT mitochondrial oxidation by Caco-2 cells.

Finally, it should be mentioned that Caco-2 cells derive from a human colon adenocarcinoma, but when fully differentiated they form a polarized monolayer of cells expressing a combination of colonocyte and enterocyte phenotypes (65). This underscores the need to further analyze the effect of polyphenols upon BT uptake and upon its anticarcinogenic effect in other human colon adenocarcinoma cell lines.

In conclusion, changes in uptake of BT induced by polyphenols do not correlate with changes on the effect of BT upon cell viability, cell proliferation, differentiation, and apoptosis, supporting the conclusion that there is no modulation of those effects of BT by the polyphenols tested.

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VI - Effect of polyunsaturated fatty acids and bile salts on butyrate uptake by intestinal epithelial cells

EFFECT OF POLYUNSATURATED FATTY ACIDS AND BILE SALTS ON BUTYRATE UPTAKE BY INTESTINAL EPITHELIAL CELLS

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ABSTRACT

Our aim was to investigate the effect of *n*-3 PUFAs (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)), *n*-6 PUFAs (linoleic acid (LA), γ-linolenic acid (LNA) and arachidonic acid (AA)), conjugated linoleic acid (CLA) and the bile salts deoxycholic acid (DCA) and taurodeoxycholic acid (TDCA) upon uptake of ^{14}C-butyrate (^{14}C-BT) by intestinal epithelial cells.

Caco-2 cells were exposed to LA, LNA, AA, DHA, EPA, CLA and DCA (100 µM) for 1 to 7 days. The only significant effects were slight (7-18%) increases in ^{14}C-BT uptake found with EPA, CLA, AA and DCA. IEC-6 cells were exposed to LA, LNA, AA, DHA, EPA, CLA, DCA and TDCA (100 µM) for 2 days. LNA, EPA, CLA and DHA caused small (9-16%) decreases in ^{14}C-BT uptake. For CLA, this effect was associated with a decrease in cellular viability.

A discrete interference of *n*-3 and *n*-6 PUFAs, CLA and bile acids with the apical uptake of BT was observed.

Keywords: bile salts; butyrate uptake; Caco-2 cells; IEC-6 cells; polyunsaturated fatty acids

INTRODUCTION

Colorectal cancer (CRC) is one of the most common solid tumours worldwide, being the second leading cause of cancer death among men and women combined in the United States, Australia and Europe, and a major problem in many other countries. The causes of CRC are multifactorial, but an association between reduced risk of CRC and a diet high in fruit, vegetables and fiber has been well established in epidemiological studies (Martínez *et al*. 2008).

Butyrate (BT), a short-chain fatty acid produced by anaerobic bacterial fermentation of dietary fibre within the human colon, plays a key role in colonic epithelium homeostasis. One of the proposed beneficial effects of BT on human colonic health is the prevention/inhibition of colon carcinogenesis (Hamer *et al*. 2008). Many epidemiological studies show an inverse relationship between dietary fibre intake and the incidence of CRC (Martínez *et al*. 2008), and exposure of many colon tumour cell lines to BT leads to anticarcinogenic effects by induction of cell differentiation and apoptosis and by inhibition of proliferation (Heerdt *et al.*1994; Hague *et al*. 1995). At the molecular level, the anticarcinogenic effect of BT is considered to depend on regulation of gene expression, which is mainly attributed to its inhibition of histone deacetylase, resulting in hyperacetylation of histones and enhancement of the accessibility of transcription factors to nucleosomal DNA (Heerdt *et al.*1994; Hague *et al*. 1995).

BT is transported into colonic epithelial cells by two specific carrier-mediated transport systems, the monocarboxylate transporter 1 (MCT1) and the sodium monocarboxylate transporter 1 (SMCT1)

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(Gupta *et al.* 2006; Morris and Felmlee 2008). Interestingly enough, both MCT1 and SMCT1 were recently proposed to function as tumour suppressors (Cuff *et al*. 2005; Gupta *et al*. 2006). So, interference with either MCT1- and SMCT1-mediated transport of BT into colonocytes will obviously have a major impact in the context of colon carcinogenesis. In this context, MCT1-mediated transport at the intestinal level is known to be up-regulated by its substrate, BT (Cuff *et al.* 2002), enhanced by leptin (Buyse *et al*. 2002), phorbol 12-myristate 13-acetate (Alrefai *et al*. 2004), caffeine and acetylsalicylic acid (Gonçalves *et al*. 2009), and inhibited by enteropathogenic *E. coli* (Borthakur *et al*. 2006), interferon-γ, tumour necrosis factor-α (Thibault *et al*. 2007), theophylline, tetrahydrocannabinol, MDMA (ecstasy), acetaldehyde and indomethacin (Gonçalves *et al*. 2009), and by some polyphenols (Konishi *et al*. 2003; Vaidyanathan and Walle 2003; Wang and Morris 2007; Shim *et al.* 2007).

n-3 (n-3) and n-6 (n-6) polyunsaturated fatty acids (PUFAs) are essential fatty acids necessary for human health. The most widely studied effects of PUFAs are those related to eicosanoid biosynthesis and function. In general terms, eicosanoids derived from n-6 PUFAs have pro-inflammatory whereas those derived from n-3 precursors have anti-inflammatory effects. Likewise, eicosanoids derived from these two series have opposing effects in cancer cell growth, invasion, and angiogenesis (Berquin et al. 2008; Chapkin et al. 2008). More specifically, in relation to CRC, and from a dietary perspective, a growing body of experimental, epidemiological and clinical evidence supports the contention that fish oil and its bioactive food components (n−3 PUFAs (eg. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are important in suppressing CRC (although several clinical trials and epidemiological studies are inconsistent), and that the opposite effect is observed with n-6 PUFAs (eg., linoleic acid (LA) and arachidonic acid (AA)), which are found in meat and vegetable oils (Berquin et al. 2008; Chapkin et al. 2008).

Conjugated linoleic acid (CLA) is a collective term for isomers of LA that have conjugated double bonds. CLA has been shown to have a number of health promoting actions, including an *in vitro* and *in vivo* anticarcinogenic effect. Among the several mechanisms proposed to be involved in its anticarcinogenic effect are alteration of lipid peroxidation, tissue fatty acid composition, eicosanoid metabolism and gene expression (Kelley *et al*. 2007).

On the other hand, secondary (unconjugated) bile acids, including deoxycholic acid (DCA), but not primary bile acids, have been shown to be tumour promoters, both *in vitro* and in animal studies (Reddy *et al*. 1977; Mahmoud *et al.* 1999). Moreover, raised levels of secondary bile acids have been reported in patients with adenomatous polyps and colon cancer (Reddy and Wynder 1977; Imray *et al*. 1992). Their mechanism of action is still poorly understood, although PKC, MAP kinases and NFκB have been identified as molecular targets (Huang *et al*. 1992; Pongracz *et al.* 1995; Payne *et al.* 2007).

Interestingly enough, the effects of BT and either PUFA (Hofmanová et al. 2005, 2009), CLA (Nichenametla et al. 2004), or bile acids (McMillan et al. 2000; Rosignoli et al. 2008) upon colonic epithelial cell population may be mutually influenced. So, the aim of this study was to investigate the hypothesis that interference with the apical uptake of BT may be one of the mechanisms contributing to the effect of n-3 PUFA, n-6 PUFA, CLA and bile acids upon CRC carcinogenesis. For this, we investigated the effect of n-3 PUFAs (DHA and EPA), n-6 PUFAs (LA, γ-linolenic acid (LNA) and AA), CLA, the unconjugated bile acid DCA and the conjugated bile acid taurodeoxycholic acid (TCDA) upon uptake of ^{14}C-BT by a human colonic adenocarcinoma cell line (Caco-cells) and by a rat small intestinal epithelial cell line (IEC-6 cells). Caco-2 cells derive from a human colon adenocarcinoma, but when fully differentiated they form a polarized monolayer of cells expressing a combination of colonocyte and enterocyte phenotypes (Sambuy et al. 2005). On the other hand, IEC-6 cells were established from crypt of rat intestinal cells, but when grown in post-confluent culture, they develop structural changes and differentiation from a crypt cell-like to an enterocyte-like phenotype (Wood et al. 2003). Comparison between the effect of these compounds upon BT uptake in carcinogenic and noncarcinogenic cell lines seemed interesting in the context of the distinct effect of BT in these cells (Hamer et al. 2008).

MATERIALS AND METHODS

Caco-2 Cell Culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage number 51-64. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma). Culture medium was changed every 2- 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and sub-cultured in plastic culture dishes (21-cm^2; ∅ 60 mm; Corning Costar, Corning, NY, USA). For uptake studies, Caco-2 cells were seeded on 24-well plastic cell culture clusters (2-cm^2; ∅ 16 mm; Corning Costar), and ^{14}C-BT uptake experiments were performed 7 days after the initial seeding. For 24 h before the experiments, the cell medium was made free of fetal calf serum.

IEC-6 Cell Culture

The IEC-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-111, Braunschweig, Germany) and was used between passage number 35-37. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were cultured in Dulbecco's Modified Eagle's Medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma). Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.05% trypsin-EDTA, 5 min, 37°C), split 1:3, and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm; Corning Costar, Corning, NY, USA). For uptake studies, IEC-6 cells were seeded on 24-well plastic cell culture clusters (2 cm^2 Ø 16 mm; Corning Costar), and ^{14}C-BT uptake experiments were performed 9 days after the initial seeding (90-100% confluence). For 24 h before the experiments, the cell medium was made free of fetal calf serum and insulin.

Determination of ^{14}C-BT uptake by Caco-2 and IEC-6 Cells

The uptake experiments were performed with Caco-2 and IEC-6 cells incubated in glucose-free Krebs buffer (containing, in mM: 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 NaHCO3, 1.6 KH_2PO_4, 0.4 K_2HPO_4, and 20 MES (pH 6.5)). Initially, the culture medium was aspirated and the cells were washed twice with 0.5 ml buffer at 37°C. Then, uptake was initiated by the addition of 0.3 ml medium at 37°C containing ^{14}C-BT 10 µM. Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold buffer. The cells were then solubilised with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris.HCl, pH 7.4), and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Treatment of the Cells with Compounds

The effect of compounds on ^{14}C-BT uptake was tested by cultivating cells in culture medium containing these compounds (or the respective solvent) for 1, 2, 3 or 7 days. The medium was

renewed daily, and the end of the treatment period was day 7 and 9 of Caco-2 and IEC-6 cell culture, respectively.

Determination of Cell Viability

To test whether the compounds affected cellular viability, the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed. In brief, cells were treated with the compounds as described above, and at the end of that period, the MTT assay was done as described elsewhere (Gonçalves *et al*. 2009).

Protein Determination

The protein content of cell monolayers was determined as described elsewhere (Bradford 1976), using human serum albumin as standard.

Calculation and Statistics

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. Statistical significance of the difference between two groups was evaluated by the Student's t-test. Differences were considered to be significant when $P<0.05$.

Materials

^{14}C-BT (*n*-butyric acid, sodium salt, [1-^{14}C]; specific activity 30-60 mCi/mmol (Biotrend Chemikalien GmbH, Köln, Germany); MES (2-[N-morpholino]ethanesulfonic acid) hydrate), arachidonic acid, docosahexaenoic acid, linoleic acid, γ-linolenic acid, eicosapentahenoic acid, conjugated linoleic acid (mixture of cis- and trans-9,11- and -10,12-octadecadienoic acids), deoxycholic acid, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), taurodeoxycholic acid, trypsin-EDTA solution (Sigma, St. Louis, MO, USA); DMSO (dimethylsulfoxide), triton X-100 (Merck, Darmstadt, Germany); fetal calf serum (Invitrogen Corporation, Carlsbad, CA, USA).

Drugs to be tested were dissolved in ethanol or DMSO, the final concentration of the solvents being 1% in the culture media. Controls were run in the presence of the solvent.

RESULTS

Previously, our group have shown that ^{14}C-BT apical uptake in Caco-2 and IEC-6 cells was found to be linear with time for up to 3 min of incubation (Gonçalves et al. 2009, 2010). So, in the present work, cells were incubated with ^{14}C-BT for 3 min, in order to determine initial rates of uptake.

Effect of Pufas and Bile Acids on ^{14}C-BT Apical uptake by Caco-2 Cells

The effect of several distinct PUFAs on ^{14}C-BT apical uptake by Caco-2 cells was investigated in the first series of experiments. For this, 100 µM of each fatty acid was tested over different

periods (1, 2, 3 and 7 days). Of the fatty acids tested, LA, LNA acid and DHA were devoid of significant effect (results not shown; *n*=6-9). However, AA, EPA and CLA caused small but significant increases in ^{14}C-BT uptake at some of the time points analysed (Fig. 1A).

In the next series of experiments, we further investigated the effect of AA and EPA, by characterizing its concentration-dependency. When tested for 2 days, and contrary to what was found with 100 µM, higher concentrations of AA and EPA decreased uptake of ^{14}C-BT (1 mM of AA or EPA reduced uptake by 20%; Fig. 1B). This decrease was associated with a decrease in cellular viability (to 65.6±1.7 and 78.6±0.8% of control, respectively; *n*=10). Interestingly enough, treatment with AA (300 µM) for 7 days, which caused no decrease in ^{14}C-BT uptake (Fig. 1B), was associated with a 24.3±6.6% increase in Caco-2 viability (*n*=10). None of these treatments (1 mM of AA or EPA for 2 days or 300 µM AA for 7 days) affected Caco-2 cell proliferation (results not shown).

The effect of the bile acid DCA (100 µM) was also investigated. Apart from a small increase found after 2 days of treatment (to 109.4±2.5% of control; *n*=9), DCA was devoid of significant effect on ^{14}C-BT uptake (results not shown).

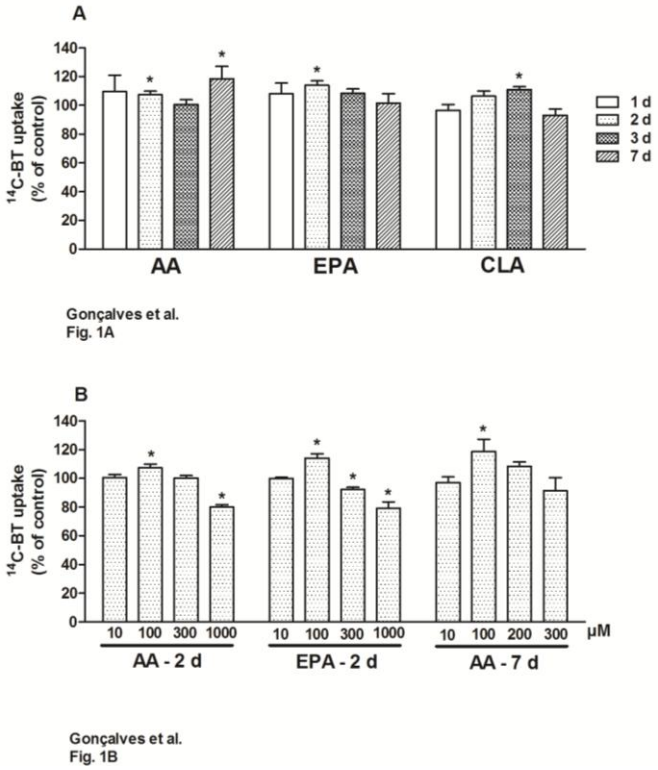

Figure 1. Effect of PUFA on ^{14}C-BT uptake by Caco-2 cells. A. Time-dependence of the effect of PUFAs on the apical uptake of ^{14}C-BT. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with 10 µM ^{14}C-BT after treatment for 1, 2, 3 or 7 days with arachidonic acid 100 µM (AA; n=9), eicosapentaenoic acid 100 µM (EPA; n=9-15) or conjugated linoleic acid 100 µM (CLA; n=9-13); B. Concentration-dependency of the effect of PUFAs on the apical uptake of ^{14}C-BT. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with 10 µM ^{14}C-BT after treatment for 2 days with increasing concentrations of arachidonic acid or eicosapentaenoic acid (AA - 2 days and EPA - 2 days, respectively; n=9) or after treatment for 7 days with increasing concentrations of arachidonic acid (AA; n=7-9). Shown are arithmetic means±SEM. * significantly different from control.

Effect of Pufas and Bile Acids on ^{14}C-BT Apical uptake by IEC-6 Cells

We then investigated the effect of distinct PUFAs on ^{14}C-BT uptake by IEC-6 cells. For this, IEC-6 cells were treated with 100 µM of each fatty acid for 2 days. Of the compounds tested, LA and AA were devoid of significant effect (results not shown; *n*=9). On the other hand, LNA, EPA, CLA and DHA caused small (9-16%) but significant decreases in ^{14}C-BT uptake (Fig. 2A). The

effect of LNA and EPA upon ^{14}C-BT uptake was found to be concentration-dependent, but this was not observed with DHA (Fig. 2B).

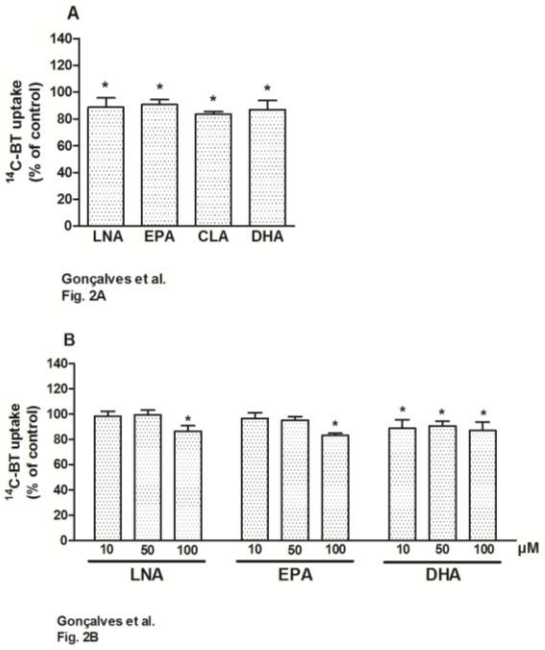

Figure 2. Effect of PUFAs on the apical uptake of ^{14}C-BT by IEC-6 cells. A. Initial rates of uptake were determined in IEC-6 cells incubated for 3 min with 10 μM ^{14}C-BT after treatment for 2 days with γ-linolenic acid 100 μM (LNA; n=6), eicosapentaenoic acid 100 μM (EPA; n=9), conjugated linoleic acid 100 μM (CLA; n=9), or docosaenoic acid (DHA; n=9); B. Concentration-dependency of the effect of PUFAs on the apical uptake of ^{14}C-BT. Initial rates of uptake were determined in IEC-6 cells incubated for 3 min with 10 μM ^{14}C-BT after treatment for 2 days with increasing concentrations of γ-linolenic acid (LNA; n=6-9), eicosapentaenoic acid (EPA; n=9) or docosahexaenoic acid (DHA; n=3-9). Shown are arithmetic means±SEM. * significantly different from control.

Interestingly enough, the inhibitory effect of 100 μM LNA, EPA and DHA was not associated with a decrease in cellular viability, as these compounds increased cellular viability (by 14.8±1.1, 21.8±1.1 and 21.9±2.4%, respectively; *n*=8-9). For CLA, however, the decrease in ^{14}C-BT uptake was associated with a similar decrease in cell viability (to 89.7±0.4% of control; *n*=8).

Finally, the effect of a 2-day exposure to the bile acids DCA and TCDA (100 μM) was also investigated. None of the compounds affected ^{14}C-BT uptake by IEC-6 cells (results not shown).

CONCLUSION

The majority of studies in the literature concerning the effects of BT, PUFAs, CLA and bile acids in colon carcinogenesis have considered these dietary factors individually. *In vivo*, however, they are commonly present together in the colon and so they may influence each other's actions directly or indirectly. For this reason, we decided to test the effect of *n*-3 PUFAs (DHA and EPA), *n*-6 PUFAs (LA, LNA and AA), CLA and the bile acids DCA and TDCA upon uptake of ^{14}C-BT by a human colonic adenocarcinoma (Caco-2) cell line and by a rat small intestinal epithelial (IEC-6) cell line. BT plays an essential role in the maintenance of colonic tissue homeostasis, and one of its proposed beneficial effects is the prevention/inhibition of colon carcinogenesis (Heerdt *et al.* 1994; Hague *et al.* 1995). So, understanding of the regulation of its absorption by the colon mucosa appears to be particularly important in the context of carcinogenesis.

Exposure of Caco-2 cells (from 1 to 7 days) to LA, LNA and DHA (100 μM) did not affect ^{14}C-BT uptake. In contrast, exposure to 100 μM EPA and DCA (for 2 days), CLA (for 3 days) or AA (for 2 and 7 days) caused small (7-18%) but significant increases in ^{14}C-BT uptake. Treatment of the

cells for 2 days with higher concentrations of either AA or EPA (1000 µM) caused a 20% decrease in ^{14}C-BT uptake, which was however associated with a parallel decrease in cellular viability. These latter results suggest that the reduction of ^{14}C-BT uptake found with the higher concentrations of either AA or EPA is related to cell death. Further support for this conclusion was the observation that treatment with a high concentration of AA (300 µM) for 7 days did not affect ^{14}C-BT uptake, and was associated with a 25% increase in Caco-2 viability.

Exposure of IEC-6 cells for 2 days to LA, AA, DCA and TDCA (100 µM) did not affect ^{14}C-BT uptake. In contrast, exposure to LNA, EPA, CLA or DHA (100 µM) caused small (9-16%) but significant decreases in ^{14}C-BT uptake. The decrease in ^{14}C-BT uptake obtained with CLA (100 µM) was associated with a parallel decrease in cellular viability. However, LNA, EPA and DHA decreased ^{14}C-BT uptake while increasing cellular viability.

In summary, ^{14}C-BT uptake is modestly modulated by some PUFA and bile salts, independently of an effect on cell viability. In Caco-2 cells, EPA, AA, CLA and DCA (100 µM) increased uptake, and in IEC-6 cells LNA, EPA and DHA (100 µM) decreased it.

In Caco-2 cells, ^{14}C-BT uptake was slightly increased by compounds possessing either anticarcinogenic (EPA, CLA) or carcinogenic (AA, DCA) activity against CRC. The same was observed in IEC-6 cells, in which ^{14}C-BT uptake was slightly decreased by compounds possessing either anticarcinogenic (EPA, DHA) or carcinogenic (LNA) activity against CRC. This strongly suggests that modulation of ^{14}C-BT uptake is not involved in the effect of these compounds on CRC. In other words, given the anticarcinogenic effect of BT in CRC cells, the increase in ^{14}C-BT uptake in Caco-2 cells caused by EPA and CLA can be one of the mechanisms contributing to their anticarcinogenic effect against CRC, but cannot contribute to the carcinogenic effect of AA and DCA. On the other hand, in IEC-6 cells, the decrease in ^{14}C-BT uptake observed with LNA can contribute to the carcinogenic effect of this PUFA in relation to CRC, but cannot contribute to the anticarcinogenic effect of EPA and DHA.

We conclude that a discrete interference of *n*-3 and *n*-6 PUFAs, CLA and bile acids with the apical uptake of BT was observed. So, this does not seem to be one of the main mechanisms involved in the recognized role that these compounds have in colon carcinogenesis.

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VII - Inhibition of butyrate uptake by the primary bile salt chenodeoxycholic acid in intestinal epithelial cells

ARTICLE
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Inhibition of Butyrate Uptake by the Primary Bile Salt Chenodeoxycholic Acid in Intestinal Epithelial Cells

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ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers worldwide. Epidemiological and experimental studies suggest that bile acids may play a role in CRC etiology. Our aim was to characterize the effect of the primary bile acid chenodeoxycholic acid (CDCA) upon ^{14}C-BT uptake in tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines. A 2-day exposure to CDCA markedly and concentration-dependently inhibited ^{14}C-BT uptake by IEC-6 cells ($IC_{50} = 120\ \mu M$), and, less potently, by Caco-2 cells ($IC_{50} = 402\ \mu M$). The inhibitory effect of CDCA upon ^{14}C-BT uptake did not result from a decrease in cell proliferation or viability. In IEC-6 cells: (1) uptake of ^{14}C-BT involves both a high-affinity and a low-affinity transporter, and CDCA acted as a competitive inhibitor of the high-affinity transporter; (2) CDCA inhibited both Na^+-coupled monocarboxylate cotransporter 1 (SMCT1)- and H^+-coupled monocarboxylate transporter 1 (MCT1)-mediated uptake of ^{14}C-BT; (3) CDCA significantly increased the mRNA expression level of SMCT1; (4) inhibition of ^{14}C-BT uptake by CDCA was dependent on CaM, MAP kinase (ERK1/2 and p38 pathways), and PKC activation, and reduced by a reactive oxygen species scavenger. Finally, BT (5 mM) decreased IEC-6 cell viability and increased IEC-6 cell differentiation, and CDCA (100 μM) reduced this effect. In conclusion, CDCA is an effective inhibitor of ^{14}C-BT uptake in tumoral and non-tumoral intestinal epithelial cells, through inhibition of both H^+-coupled MCT1- and SMCT1-mediated transport. Given the role played by BT in the intestine, this mechanism may contribute to the procarcinogenic effect of CDCA at this level. J. Cell. Biochem. 113: 2937–2947, 2012.

KEY WORDS: BUTYRATE UPTAKE; CHENODEOXYCHOLIC ACID; IEC-6 CELLS; Caco-2 CELLS; MCT1; SMCT1

Colorectal cancer (CRC) is a leading cause of cancer death in occidental countries [Jemal et al., 2011]. The causes of CRC are multifactorial, and a good correlation between a diet high in saturated fats and low in dietary fiber, fruit, and vegetables, and an increased risk of CRC has been well established by epidemiological studies [Martínez et al., 2008].

Short-chain fatty acids [(SCFAs) acetate, propionate, and butyrate (BT)] are organic acids produced in the intestinal lumen by bacterial fermentation of mainly undigested dietary fiber. Among SCFA, BT plays a key role in colonic epithelium homeostasis, by having multiple regulatory roles at that level, including: (1) being the main energy source for colonocytes; (2) inhibition of colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation); (3) promotion of growth and proliferation of normal colonic epithelial cells; (4) stimulation of fluid and electrolyte absorption; (5) stimulation of mucus secretion and increase of vascular flow and motility; (6) reduction of visceral perception, intestinal discomfort, and pain; (7) inhibition of colon inflammation and oxidative stress; and (8) improvement of the colonic defense barrier function [Hamer et al., 2008].

As stated, one of the proposed beneficial effects of BT on human colonic health is the prevention/inhibition of colon carcinogenesis

Abbreviations used: ALP, alkaline phosphatase; BT, butyrate; Caco-2, human epithelial colon adenocarcinoma cell line; CaM, Ca^{2+}/calmodulin; CaMK II, CaM-dependent protein kinase II; CDCA, chenodeoxycholic acid; CRC, colorectal cancer; IEC-6, rat non-tumoral small intestinal epithelial cell line; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MCT1, H^+-coupled monocarboxylate transporter 1; MTT, 3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PTK, protein tyrosine kinases; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; SCFA, short-chain fatty acid; SMCT1, Na^+-coupled monocarboxylate cotransporter 1; SRB, sulforhodamine B.

No conflicts of interest are declared by the authors.

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[Hamer et al., 2008]. BT is transported into intestinal epithelial cells by two specific carrier-mediated transport systems, the electroneutral H^+-coupled monocarboxylate transporter 1 (MCT1) [Cuff et al., 2005] and the Na^+-coupled monocarboxylate cotransporter 1 (SMCT1) [Gupta et al., 2006]. MCT1 [Cuff et al., 2005] and SMCT1 [Gupta et al., 2006] were recently proposed to function as tumor suppressors, most probably due to their ability to mediate the entry of BT into intestinal epithelial cells. Therefore, factors that interfere with BT uptake into intestinal epithelial cells are potentially detrimental to intestinal health and integrity by promoting CRC.

Primary bile acids, predominantly cholic acid and chenodeoxycholic acid (CDCA), are produced in the liver by the metabolism of cholesterol and are delivered into the intestinal tract as glycine or taurine conjugates. Most bile acids are reabsorbed in the ileum as conjugates. However, during each enterohepatic cycle, about 10% of the bile acids escape into the colon where they are exhaustively converted by the intestinal flora, and 1–3% of the bile acids will eventually be excreted in the feces. Fecal bile acids are almost completely deconjugated and have undergone several other reactions, such as dehydroxylation, dehydrogenation, and epimerization [Ridlon et al., 2006]. Intestinal epithelial cells are thus exposed to various concentrations and compositions of bile acids.

Several epidemiological studies have found that fecal bile acid concentrations are elevated in populations with a high incidence of CRC [Reddy et al., 1977, 1978], and high-fat, high-cholesterol diets, which increase the risk of CRC, also increase the total of bile acids in the gut [Stadler et al., 1988]. In agreement with these observations, secondary bile acids have been shown to be tumor promoters, both in vitro and in animal studies [Reddy et al., 1977; Mahmoud et al., 1999]. Their mechanism of action is still poorly understood, although PKC, MAP kinases (MAPK), and nuclear factor-kappa B (NF-κB) were identified as molecular targets for these compounds [Qiao et al., 2000; McMillan et al., 2003].

In relation to primary bile acids, less information is available. However, a significantly higher fecal concentration of the primary bile salt CDCA was seen in patients with CRC/adenoma [Tong et al., 2008] and CDCA was also shown to be tumor promoting in some animal and cell culture studies [Mahmoud et al., 1999; McMillan et al., 2003].

The majority of studies concerning the effects of BT and bile acids on intestinal epithelial cells have considered these factors individually. However, in vivo, both are present in the colon and may directly or indirectly influence each other's actions. Very recently, we demonstrated a discrete interference of the bile salt deoxycholic acid upon uptake of ^{14}C-BT by intestinal epithelial cells [Gonçalves et al., 2011d]. So, the aim of the present study was to investigate the hypothesis that interference with the apical uptake of BT may be one of the mechanisms contributing to the effect of the primary bile acid CDCA upon colon carcinogenesis.

MATERIALS AND METHODS

CACO-2 AND IEC-6 CELL CULTURE

The Caco-2 and IEC-6 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passage numbers 53–58 and 17–30, respectively. The cells were maintained in a humidified atmosphere of 5% CO_2–95% air. Caco-2 cells were cultured in minimum essential medium (Sigma, St. Louis, MO) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). IEC-6 cells were cultured in Dulbecco's modified Eagle's medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split 1:3, and sub-cultured in plastic culture dishes (21 cm^2; Ø 60 mm; Corning Costar, Corning, NY). For use in experiments, cells were seeded on 24-well plastic cell culture clusters (2 cm^2 Ø 16 mm; TPP®), and experiments were performed 7–9 days after the initial seeding (90–100% confluence).

TREATMENT OF THE CELLS

The effect of CDCA was tested by cultivating cells in culture medium containing CDCA (or the respective solvent) for 1, 2, 3, or 7 days. The effect of BT was tested by cultivating cells in culture medium containing BT or the respective solvent for 2 days. The effect of inhibitors of signaling pathways was tested by cultivating cells in culture medium containing CDCA, these compounds or the respective solvents for 1 day. The medium was renewed daily and the end of the treatment period was always days 7–9 of cell culture.

TRANSPORT STUDIES

Transport experiments were performed with Caco-2 and IEC-6 cells incubated in glucose-free Krebs (GFK) buffer containing (mM): 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4, and 20 MES, pH 6.5 [Gonçalves et al., 2009]. Initially, the culture medium was aspirated and the cells were washed with 0.3 ml GFK buffer at 37°C. Then, uptake was initiated by the addition of 0.3 ml GFK buffer at 37°C containing ^{14}C-BT (10 μM, except in kinetic experiments). Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold GFK buffer. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM, Tris–HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting. In the Na^+ dependence experiments, NaCl was substituted by an isotonic concentration of LiCl.

DETERMINATION OF CELLULAR VIABILITY

Two different methods were used to determine cell viability.

MTT (3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide) assay. After 45 h of treatment, 50 μl MTT solution (5 mg/ml) were added to each well. The cells were then further incubated for 3 h at 37°C. The formazan crystals derived from MTT cleavage were then measured as described by Mosmann [1983].

Lactate dehydrogenase (LDH) assay. After the treatment period (48 h), cellular leakage of the cytosolic enzyme LDH into the extracellular (culture) medium was measured spectrophotometrically, by quantification of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described by Bergmeyer and Bernt [1974].

DETERMINATION OF CELLULAR PROLIFERATION [SULFORHODAMINE B (SRB) ASSAY]

After the period of treatment (48 h), the whole cell protein was determined, as described previously by our group [Gonçalves et al., 2011c].

DETERMINATION OF CELLULAR DIFFERENTIATION (ALKALINE PHOSPHATASE ACTIVITY ASSAY)

After the period of treatment (48 h), cell differentiation was measured by quantification of alkaline phosphatase (ALP) activity, as previously described [Gonçalves et al., 2011a]. ALP activity was determined spectrophotometrically, by using *p*-nitrophenylphosphate as substrate. The results are expressed as nmol *p*-nitrophenol/min/mg protein.

QRT-PCR

Total RNA was extracted from IEC-6 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Germany). cDNA synthesis and real-time PCR was carried out as described by Gonçalves et al. [2011abc]. Annealing temperatures (AT) and sequence of primers are indicated in Table I. Data were analyzed using LightCycler® 4.05 analysis software (Roche, Mannheim, Germany).

PROTEIN DETERMINATION

The protein content of cell monolayers was determined as described by Bradford [1976], using human serum albumin as standard.

CALCULATION AND STATISTICS

For the analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis–Menten equation were fitted to the experimental data by a nonlinear regression analysis using a computer-assisted method [Muzyka et al., 2005].

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. Statistical analysis of the difference between various groups was evaluated by the ANOVA test, followed by the Student–Newman–Keuls test. Statistical analysis of the difference between two groups was evaluated with Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

TABLE I. Primers Used in qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
rGAPDH	F: GGC ATC GTG GAA GGG CTC ATG AC R: ATG CCA GTG AGC TTC CCG TTC AGC	62
rMCT1	F: CAG TGC AAC GAC CAG TGA ATG TG R: ATC AAG CCA CAG CCA GAC AGG	69
rSMCT1	F: CGG GAT CAC CAG CAC CTA C R: GCA GGG GCA TAA ATC ACA ATC	62

rGAPDH, rat glyceraldehyde-3-phosphate dehydrogenase; rMCT1, rat monocarboxylate transporter type 1; rSMCT1, rat Na^+-coupled monocarboxylate transporter type 1; F, forward; R, reverse.

MATERIALS

^{14}C-BT ([1-^{14}C]-*n*-butyric acid, sodium salt; specific activity 30–60 mCi/mmol; Biotrend Chemikalien GmbH, Köln, Germany); 4′,5,7-trihydroxyisoflavone (genistein), 5,5′-dimetil-BAPTA-AM, antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), calmidazolium, chelerythrine chloride, H7, H-89 dihydrochloride hydrate, KN-62, MES (2-[*N*-morpholino]ethanesulfonic acid hydrate), nicotinamide adenine dinucleotide (NADH), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4-(hydroxymercuri)benzoic acid sodium salt (pCMB), *p*-nitrophenylphosphate, serum albumin, sodium BT, sodium chenodeoxycholate (CDCA), SRB, trichloroacetic acid sodium salt, trypsin–EDTA solution (Sigma); fetal calf serum (Invitrogen Corporation, Carlsbad, CA); PD 98058 and SB 203580 (Research Biochemicals International, Natick); dimethylsulfoxide (DMSO), triton X-100 (Merck, Darmstadt, Germany).

Drugs to be tested were dissolved in water, DMSO, or ethanol; the final concentration of these solvents in the culture medium and GFK buffer was 1% and 0.1%, respectively. Controls for these drugs were run in the presence of the respective solvent.

RESULTS

TIME- AND CONCENTRATION-DEPENDENCE OF THE EFFECT OF CDCA UPON ^{14}C-BT APICAL UPTAKE IN CACO-2 AND IEC-6 CELLS

Our group had previously shown that the apical uptake of ^{14}C-BT in both Caco-2 cells [Gonçalves et al., 2009] and IEC-6 cells [Gonçalves et al., 2011b] was linear with time for up to 3 min of incubation. So, in the present work, cells were incubated with ^{14}C-BT for 3 min in order to measure initial rates of uptake.

The effect of CDCA upon ^{14}C-BT apical uptake by Caco-2 and IEC-6 cells was investigated in a first series of experiments. For this, CDCA (100 μM) was tested over different time periods (1, 2, 3, and 7 days). As shown in Figure 1A, CDCA was devoid of a significant effect upon the apical uptake of ^{14}C-BT in Caco-2 cells. By contrast, in IEC-6 cells, CDCA strongly decreased (≈50%) ^{14}C-BT apical uptake, in a time-independent manner up to 7 days of exposure (Fig. 1B).

We further investigated the effect of a 2-day treatment with CDCA upon the apical uptake of ^{14}C-BT, by characterizing its concentration-dependency. As shown in Figure 2, CDCA inhibited ^{14}C-BT uptake in both Caco-2 and IEC-6 cells in a concentration-dependent manner, but with a higher potency in IEC-6 cells. Its IC_{50} was found to be 402 (255–634) μM and 120 (88–164) μM in Caco-2 and IEC-6 cells, respectively.

EFFECT OF CDCA UPON THE KINETICS OF ^{14}C-BT APICAL UPTAKE IN IEC-6 CELLS

Next, we decided to investigate the effect of a 2-day exposure of IEC-6 cells to CDCA (100 μM) upon the kinetic parameters of ^{14}C-BT

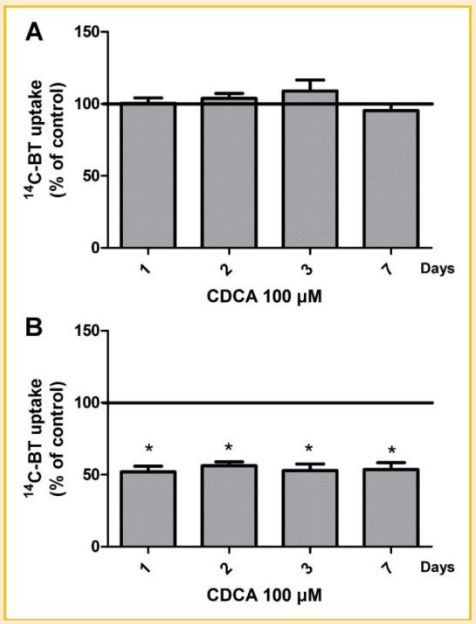

Fig. 1. Time-dependence of the effect of chenodeoxycholic acid (CDCA; 100 μM) upon the apical uptake of ^{14}C-BT by Caco-2 cells (A) and IEC-6 cells (B). Initial rates of uptake were determined in cells incubated for 3 min with 10 μM ^{14}C-BT after treatment for 1, 2, 3, or 7 days with CDCA 100 μM ($n = 6$–9). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$).

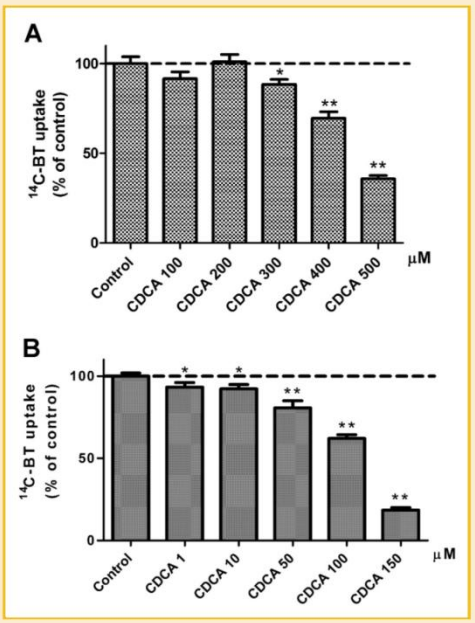

Fig. 2. Concentration-dependence of the effect of chenodeoxycholic acid (CDCA) upon the apical uptake of ^{14}C-BT by Caco-2 cells (A) and IEC-6 cells (B). Initial rates of uptake were determined in cells incubated for 3 min with 10 μM ^{14}C-BT after treatment for 2 days with CDCA ($n = 8$–16). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$). **Significantly different from the respective control ($P < 0.005$).

apical uptake. Kinetic experiments revealed the existence of a high affinity and a low-affinity transporter for ^{14}C-BT (Fig. 3). The high- and low-affinity transporters of BT in IEC-6 cells most probably correspond to SMCT1 and MCT1, respectively [Thangaraju et al., 2008; Gonçalves et al., 2011b]. We verified that CDCA was devoid of significant effect upon the kinetics of ^{14}C-BT uptake mediated by the low-affinity transporter ($V_{max} = 57.6 \pm 11.3$ and 78.0 ± 20.4 nmol mg prot^{-1} 3 min^{-1}, $K_m = 2.66 \pm 0.94$ and 3.39 ± 1.46 mM; in the absence and presence of CDCA, respectively; Fig. 3A). On the other hand, CDCA behaved as a competitive inhibitor of ^{14}C-BT apical uptake mediated by the high-affinity transporter, as it significantly increased the K_m of uptake (40.2 ± 3.7–95.4 ± 16.5 μM), without changing the V_{max} (3.91 ± 0.14 and 4.37 ± 0.42 nmol mg prot^{-1} 3 min^{-1} in the absence and presence of CDCA, respectively; Fig. 3B).

EFFECT OF CDCA UPON SMCT1 AND MCT1-MEDIATED ^{14}C-BT APICAL UPTAKE IN IEC-6 CELLS

In IEC-6 cells, Na^+-dependent BT uptake corresponds to SMCT1-mediated transport, and Na^+-independent uptake mainly corresponds to MCT1-mediated transport [Borthakur et al., 2010; Gonçalves et al., 2011b]. So, in order to distinguish between the effect of CDCA upon SMCT1- and MCT1-mediated ^{14}C-BT apical uptake, we compared the inhibitory effect of CDCA (100 μM) upon ^{14}C-BT uptake in the presence and absence of NaCl (which was substituted by LiCl). As shown in Figure 4A, about 60% of ^{14}C-BT apical uptake by IEC-6 cells is Na^+-dependent. CDCA (100 μM) caused a decrease of ^{14}C-BT uptake to ≈20%, both in the presence of NaCl or LiCl. This indicates that CDCA inhibits SMCT1-mediated ^{14}C-BT uptake and strongly suggests that it also inhibits MCT1-mediated ^{14}C-BT uptake. Confirmation that CDCA is able to inhibit MCT1-mediated ^{14}C-BT uptake was obtained by verifying that the inhibitory effect of CDCA upon Na^+-independent ^{14}C-BT uptake by IEC-6 cells disappeared in the presence of the classical MCT1 inhibitors, pCMB and NPPB (Fig. 4B,C).

REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR IN IEC-6 CELLS

A comparison between the mRNA expression levels of MCT1 and SMCT1 in control and CDCA-treated IEC-6 cells was then evaluated. Quantification of mRNA levels shows that IEC-6 express 10× more SMCT1 than MCT1, and that SMCT1 mRNA levels were significantly higher (and MCT1 mRNA levels tended to be higher) in cells treated with CDCA, in relation to control cells (≈80%; Fig. 5).

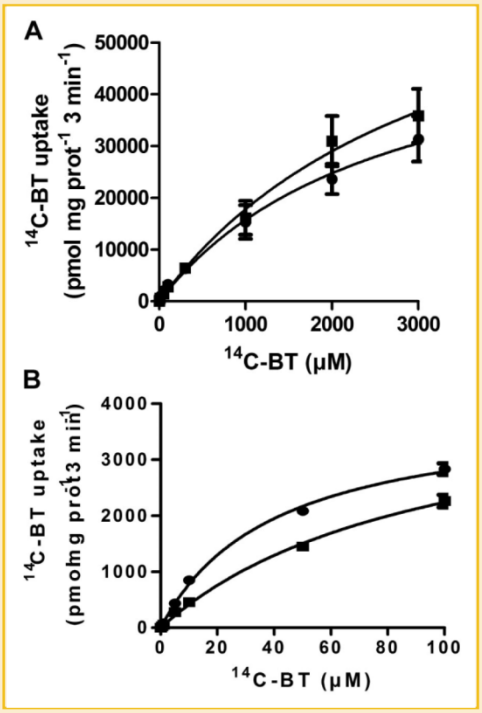

Fig. 3. Effect of chenodeoxycholic acid (CDCA; 100 μM) upon the kinetic parameters of the apical uptake of ^{14}C-BT by IEC-6 cells. Initial rates of uptake were determined in IEC-6 cells incubated at 37°C with 0.01–0.1 mM (high-affinity transport, A) or 0.01–3 mM (low-affinity transport, B) of ^{14}C-BT for 3 min, after treatment for 2 days with CDCA 100 μM (closed squares) or the respective solvent (control; closed circles; n = 4–8). Shown are arithmetic means ± SEM.

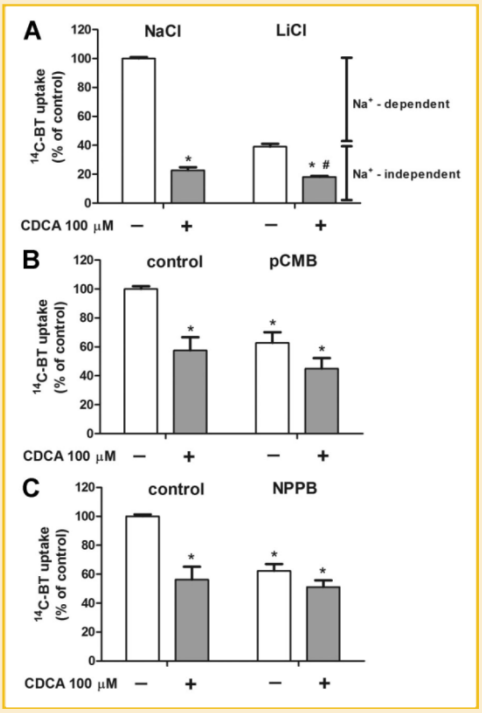

Fig. 4. Effect of chenodeoxycholic acid (CDCA; 100 μM) upon SMCT1- and MCT1-mediated apical uptake of ^{14}C-BT by IEC-6 cells. A: Effect of CDCA upon Na^+-dependent and Na^+-independent uptake. After treatment for 2 days with CDCA or the respective solvent (control), cells were incubated at 37°C with ^{14}C-BT 10 μM for 3 min in GFK buffer containing NaCl (control) or an isotonic concentration of LiCl (n = 10). B,C: Effect of CDCA and MCT1 inhibitors upon Na^+-independent uptake. After treatment for 2 days with CDCA or the respective solvent (control), cells were preincubated for 20 min and then incubated at 37°C with ^{14}C-BT 10 μM for 3 min in GFK buffer containing LiCl in the absence (control) or presence of *p*-chloromercuribenzoate (pCMB) 0.5 mM (n = 9) or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) 0.5 mM (n = 9). *Significantly different from control ($P < 0.05$). #Significantly different from CDCA ($P < 0.05$).

EFFECT OF MODULATORS OF INTRACELLULAR SIGNALING PATHWAYS ON CDCA-INDUCED INHIBITION OF ^{14}C-BT UPTAKE IN IEC-6 CELLS

We next investigated the intracellular signaling mechanisms involved in the inhibition of ^{14}C-BT uptake caused by CDCA. For this, the effect of a 1-day exposure of IEC-6 cells to modulators of intracellular signaling pathways, to CDCA or to a combination of both was assessed.

First, the role of intracellular Ca^{2+} in CDCA-induced inhibition of ^{14}C-BT uptake was investigated, by testing the effect of the Ca^{2+} chelator BAPTA-AM. No significant change in ^{14}C-BT uptake was found with BAPTA-AM, and the effect of CDCA was also not changed in the presence of this agent (Fig. 6).

We then evaluated the role of the Ca^{2+}/calmodulin (CaM) inhibitor calmidazolium, and of the CaM-dependent protein kinase II (CaMK II) inhibitor KN-62 [Said et al., 1999]. Uptake of ^{14}C-BT was significantly reduced in the presence of calmidazolium, and slightly increased in the presence of KN-62 (Fig. 6), suggesting that BT uptake is dependent on intracellular CaM. The effect of CDCA upon

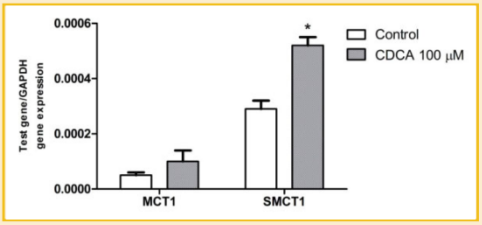

Fig. 5. Quantification of mRNA levels of MCT1 and SMCT1, by qRT-PCR, in IEC-6 cells after treatment for 2 days with chenodeoxycholic acid (CDCA; 100 μM) or its solvent (Control; n = 5). Results are shown as the expression of MCT1 or SMCT1 relative to GAPDH (arithmetic means ± SEM). *Significantly different from control ($P < 0.05$).

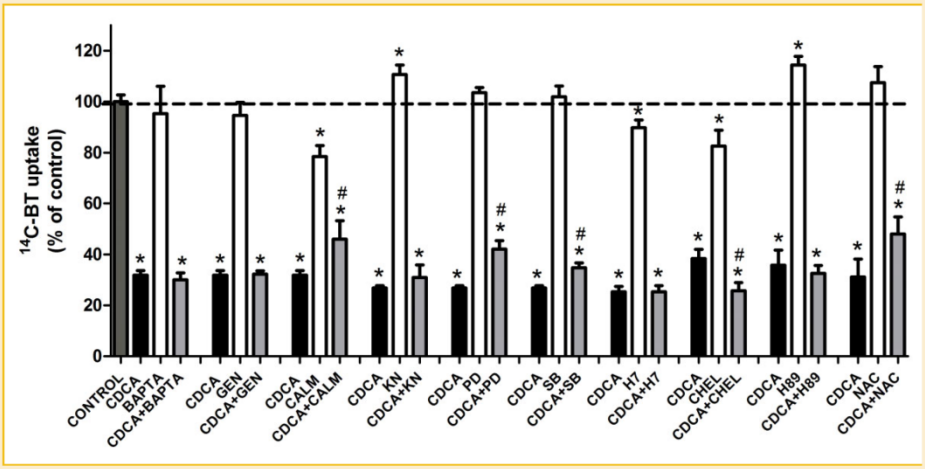

Fig. 6. Effect of inhibitors of intracellular signaling pathways upon the apical uptake of ^{14}C-BT and upon CDCA-induced inhibition of ^{14}C-BT uptake by IEC-6 cells. Initial rates of ^{14}C-BT uptake were determined in cells incubated for 3 min with ^{14}C-BT 10 μM after treatment for 1 day with CDCA 100 μM (CDCA), BAPTA AM 5 μM (BAPTA), CDCA 100 μM + BAPTA AM 5 μM (CDCA + BAPTA), genistein 10 μM (GEN), CDCA 100 μM + genistein 10 μM (CDCA + GEN), calmidazolium 0.5 μM (CALM), CDCA 100 μM + calmidazolium 0.5 μM (CDCA + CALM), KN-62 1.5 μM (KN), CDCA 100 μM + KN-62 1.5 μM (CDCA + KN), PD 98058 2.5 μM (PD), CDCA 100 μM + PD 98058 2.5 μM (CDCA + PD), SB 203580 2.5 μM (SB), CDCA 100 μM + SB 203580 2.5 μM (CDCA + SB), H-7 10 μM (H7), CDCA 100 μM + H-7 10 μM (CDCA + H7), chelerythrine 1 μM (CHEL), CDCA 100 μM + chelerythrine 1 μM (CDCA + CHEL), H-89 10 μM (H89), CDCA 100 μM + H-89 10 μM (CDCA + H89), *N*-acetyl-cysteine 1 mM (NAC), CDCA 100 μM + *N*-acetyl-cysteine 1 mM (CDCA + NAC), or the respective solvents (control) (n = 6–9). Shown are arithmetic means ± SEM. *Significantly different from the respective control ($P < 0.05$). #Significantly different from CDCA 100 μM ($P < 0.05$).

^{14}C-BT uptake was not changed in the presence of KN-62, but was reduced by calmidazolium (Fig. 6).

The involvement of MAPK was studied by testing the effect of specific inhibitors of MAPK ERK1/2 (PD 98059) [Alessi et al., 1995] and p38 MAPK (SB 203580) [Cuenda et al., 1995]. Interestingly enough, both PD 98059 and SB 203580 were devoid of effect upon ^{14}C-BT uptake, but they were able to partially reduce the inhibitory effect of CDCA upon ^{14}C-BT uptake (Fig. 6).

Next, we tested the effect of H-7, a non-selective inhibitor of PKA, PKC, and PKG [Hidaka et al., 1984]. Although H-7 was found to cause a small (≈10%) but significant decrease in ^{14}C-BT uptake, it was not able to change the effect of CDCA upon this parameter (Fig. 6). We then studied the effect of specific inhibitors of PKC (chelerythrine) and PKA (H-89) [Silva et al., 2010]. PKC inhibition was found to cause a significant decrease (≈17%) and PKA inhibition caused a significant increase (≈14%) in ^{14}C-BT uptake. This suggests that ^{14}C-BT uptake by IEC-6 cells is stimulated by PKC and inhibited by PKA activation, an observation in perfect agreement with previous publications [Alrefai et al., 2004; Narumi et al., 2010]. Interestingly enough, chelerythrine (PKC inhibitor) was able to significantly reduce the inhibition of ^{14}C-BT uptake induced by CDCA, but H89 (PKA inhibitor) had no effect (Fig. 6).

The involvement of protein tyrosine kinases (PTK) was studied by testing genistein, a known PTK inhibitor [Said et al., 1999]. As shown in Figure 7, neither ^{14}C-BT uptake nor CDCA-induced decrease of ^{14}C-BT uptake were affected by this drug (Fig. 6).

We also evaluated the putative involvement of oxidative stress in CDCA-mediated inhibition of ^{14}C-BT uptake [Song et al., 2007; Ignacio Barrasa et al., 2011], by testing the effect of the ROS scavenger *N*-acetyl-cysteine (NAC) [Aruoma et al., 1989]. NAC was devoid of effect upon ^{14}C-BT uptake but it was able to reduce the inhibitory effect of CDCA upon ^{14}C-BT uptake (Fig. 6).

The involvement of NF-κB signal transduction pathway was investigated by testing the effect of a specific inhibitor of IkBa phosphorylation and degradation (BAY 11-7082) [Pierce et al., 1997]. BAY 11-7082 (500 nM) was devoid of effect upon ^{14}C-BT uptake and did not change the inhibitory effect of CDCA upon this parameter (results not show).

Finally, the involvement of cyclooxygenases (COX) in CDCA-mediated inhibition of ^{14}C-BT uptake was tested by using quinacrine, a selective phospholipase A_2 inhibitor [Winocour et al., 1981]. Quinacrine (500 μM) was devoid of effect upon ^{14}C-BT uptake and also did not affect the inhibitory effect of CDCA upon ^{14}C-BT uptake (results not shown).

EFFECT OF CDCA ON VIABILITY AND PROLIFERATION OF IEC-6 AND CACO-2 CELLS

The effect of CDCA on cell viability and proliferation was evaluated in order to exclude these factors as responsible for its effect upon ^{14}C-BT uptake. For this, the effect of a 2-day exposure to increasing concentrations of CDCA upon Caco-2 cellular viability and upon IEC-6 cellular viability and proliferation was investigated. We verified that CDCA up to 500 μM did not affect Caco-2 cell viability

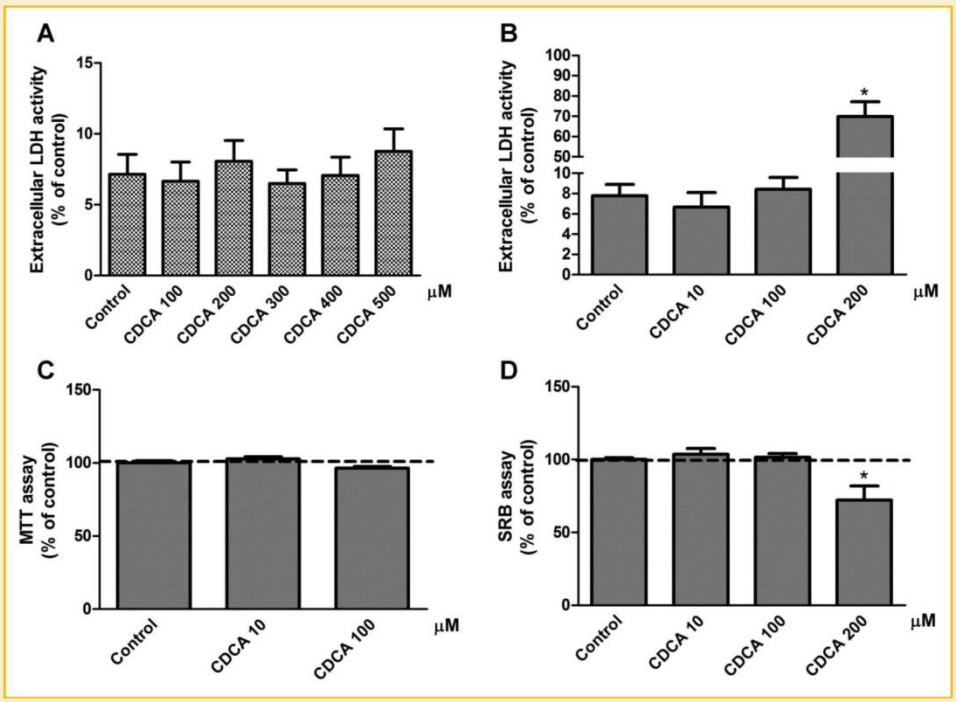

Fig. 7. Effect of a 2-day exposure to increasing concentrations of chenodeoxycholic acid (CDCA) upon Caco-2 cellular viability (A) and IEC-6 cellular viability (B,C) and proliferation (D). Cellular viability was determined by (A,B) quantification of extracellular lactate dehydrogenase activity ($n = 12–17$) and (C) the MTT assay ($n = 10$). Cell proliferation was quantified by (D) the SRB assay ($n = 6–12$). A,B: Cells were seeded on 24-well plates and cellular viability was determined by quantification of extracellular lactate dehydrogenase activity, as described in Materials and Methods Section. Results are shown as extracellular LDH activity (as % of total LDH activity). C: Cells were seeded on 24-well plates and cellular viability was determined by the MTT assay, as described in Materials and Methods Section. Results are shown as % of control. D: Cells were seeded on 24-well plates and cellular growth was determined by quantification of whole cellular protein with SRB, as described in Materials and Methods Section. Results are shown as absorbance (% of control). Results are presented as arithmetic means + SEM. *Significantly different from control ($P < 0.05$).

(Fig. 7A), and that CDCA up to 100 μM did affect neither cell viability (Fig. 7B,C) nor proliferation (Fig. 7D) of IEC-6 cells. However, 200 μM CDCA caused a significant decrease in IEC-6 cell viability (Fig. 7B) and proliferation (Fig. 7D).

THE EFFECT OF THE INTERACTION BETWEEN BT AND CDCA UPON IEC-6 CELLULAR VIABILITY, PROLIFERATION, AND DIFFERENTIATION

In this final series of experiments, we investigated whether CDCA was able to modify the effects of BT upon cell viability, proliferation, and differentiation. The effect of BT upon IEC-6 cell viability, proliferation, and differentiation was recently assessed by our group [Gonçalves et al., 2011c]. On the basis of these results, we selected BT 5 mM for further experiments.

As shown in Figure 8, BT (5 mM) caused a significant decrease in IEC-6 cell proliferation (≈20%) and viability (≈13%) and a very marked increase in cell differentiation (≈1,000%). CDCA (100 μM) did not affect cellular viability or differentiation, and caused only a small increase (≈10%) in cell proliferation. Interestingly enough, combination of CDCA with BT resulted in an attenuation of the effects of BT upon cell proliferation and differentiation. On the contrary, the effect of BT upon cell viability was potentiated by CDCA (Fig. 8).

DISCUSSION

BT plays a key regulatory role in colonic epithelium homeostasis. Therefore, factors that interfere with BT uptake into colonic epithelial cells are potentially detrimental to intestinal health and integrity. Very recently, we demonstrated a discrete interference of some n-3 polyunsaturated fatty acids (PUFAs; docosahexaenoic acid and eicosapentaenoic acid), n-6 PUFAs (linoleic acid, γ-linolenic acid, and arachidonic acid), conjugated linoleic acid, and the bile salt deoxycholic acid upon uptake of ^{14}C-BT by intestinal epithelial cells [Gonçalves et al., 2011d]. Secondary and primary bile acids have tumor promoting effects, but the

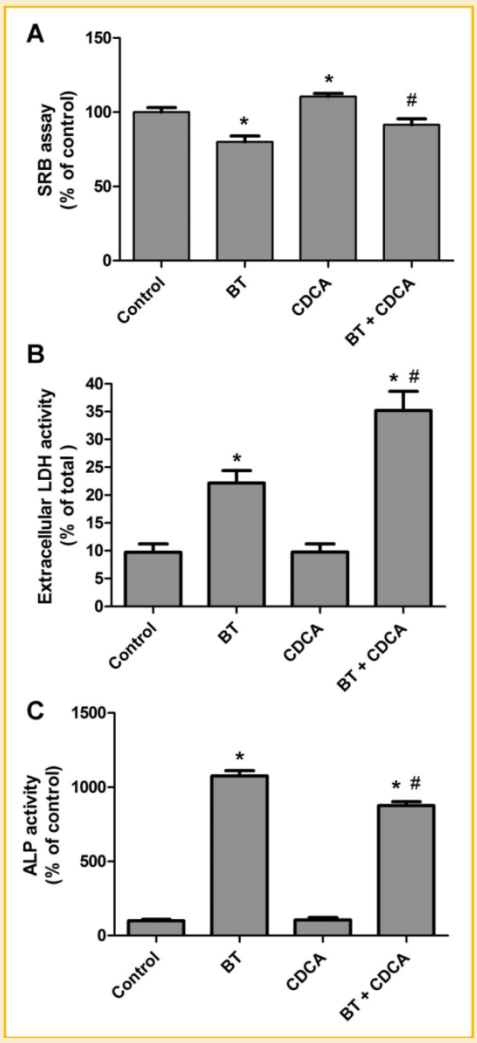

Fig. 8. Effect of a 2-day exposure to BT 5 mM (BT), chenodeoxycholic acid 100 μM (CDCA), or to a combination of both compounds (BT + CDCA) upon IEC-6 cellular proliferation (A), viability (B), and differentiation (C). A: Cellular proliferation was determined by quantification of whole cellular protein with SRB, as described in Materials and Methods Section. Results are shown as absorbance (% of control; n = 12). B: Cellular viability was determined by quantification of extracellular LDH activity, as described in Materials and Methods Section. Results are shown as extracellular LDH activity (% of total LDH activity; n = 17–18). C: Cell differentiation was determined by quantification of alkaline phosphatase (ALP) activity, as described in Materials and Methods Section. Results are shown as nmol *p*-nitrophenol/min/mg protein (% of control; n = 18). Results are presented as arithmetic means + SEM. *Significantly different from control ($P < 0.05$). #Significantly different from BT ($P < 0.05$).

mechanisms involved remain to be fully elucidated. So, the aim of the present study was to investigate the possibility that inhibition of the apical uptake of BT may be one mechanism contributing to the procarcinogenic effect of the primary bile acid CDCA at the intestinal level. We found important to compare the effect of CDCA in a colon adenocarcinoma cell line, the Caco-2 cells [Sambuy et al., 2006] and a non-tumoral intestinal epithelial cell line, the IEC-6 cells [Wood et al., 2003], as comparison between a carcinogenic and a non-carcinogenic cell line seemed interesting in the context of a possible distinct effect of CDCA in these cells. Although not a colonic cell line, the IEC-6 cells proved to be a good cellular model to study colonic BT uptake [Borthakur et al., 2010; Gonçalves et al., 2011b], because they express the BT transporters (MCT1, SMCT1, and BCRP) expressed in the human colon [Borthakur et al., 2010; Gonçalves et al., 2011b,c].

A 2-day exposure to CDCA markedly and concentration-dependently inhibited ^{14}C-BT uptake by IEC-6 cells ($IC_{50} = 120$ μM), mM), and, less potently, by Caco-2 cells ($IC_{50} = 402$ μM). The effect of CDCA in IEC-6 cells (up to 100 μM) and in Caco-2 cells (up to 500 μM) does not appear to be related to changes in cell viability or proliferation. The inhibitory effect of CDCA (100 μM) upon ^{14}C-BT uptake by IEC-6 cells was constant from 1 to 7 days of exposure, and CDCA behaved as a competitive inhibitor of the high-affinity uptake mechanism for ^{14}C-BT.

Absorption of BT from the intestinal lumen involves both the H^+-coupled MCT1 [Cuff et al., 2005] and the Na^+-coupled SMCT1 [Gupta et al., 2006], both of which are functionally present in IEC-6 cells [Borthakur et al., 2010; Gonçalves et al., 2011b]. Interestingly enough, the observation that CDCA reduced both the Na^+-dependent and the Na^+-independent uptake of ^{14}C-BT, and that inhibition of the Na^+-independent uptake of ^{14}C-BT by CDCA disappeared in the presence of MCT1 inhibitors (NPPB and pCMB) clearly show that CDCA inhibits both MCT1- and SMCT1-mediated BT uptake. It is important to note that IEC-6 cells, similarly to normal human colonic epithelium, functionally express both MCT1- and SMCT1-mediated transport, and CDCA was able to inhibit both transport mechanisms. Moreover, the lower inhibitory potency of CDCA in relation to ^{14}C-BT uptake by Caco-2 cells ($IC_{50} = 402$ μM), in which BT uptake is mainly MCT1-mediated [Gonçalves et al., 2009] and the fact that, in IEC-6 cells, CDCA (100 μM) inhibits only ^{14}C-BT uptake mediated by the high-affinity transporter (SMCT1 and MCT1 being high- and low-affinity BT transporters, respectively [Thangaraju et al., 2008]), suggests that the inhibitory effect of CDCA is more pronounced for SMCT1 than for MCT1.

Next, we evaluated the effect of CDCA on the mRNA expression levels of MCT1 and SMCT1 in IEC-6 cells. Contrary to the effect of CDCA upon ^{14}C-BT uptake, treatment of IEC-6 cells for 2 days with CDCA caused a marked (≈80%) increase in the steady-state mRNA levels of SMCT1, and a tendency to an increase in the steady-state mRNA levels of MCT1. Colchicine, an agent well known to affect the membrane trafficking of cytoplasmic proteins, did not change the effect of CDCA upon ^{14}C-BT uptake (results not show), and so CDCA appears to have no effect in the amount of transporters inserted in the cell membrane. We thus conclude that CDCA is acting possibly through decreased SMCT1 and/or MCT1 intrinsic activity, and we speculate that the increase in SMCT1 mRNA level is a compensation

mechanism in response to the marked decrease in SMCT1 activity caused by CDCA.

In relation to the mechanism(s) involved in the inhibitory effect of CDCA upon ^{14}C-BT uptake, several hypothesis can be advanced. These include: (1) changes in membrane structure, as bile acids cause the release of cell membrane components (e.g., proteins and phospholipids) prior to the occurrence of significant cell lysis [Coleman and Holdsworth, 1976]; (2) changes in membrane fluidity, as bile acids increase membrane fluidity [Zhao and Hirst, 1990], and changes of the physical state of plasma membrane lipids can influence the function of membrane carriers [Lundbaek et al., 2010]; (3) mitochondrial damage resulting in cellular ATP depletion, as this mechanism was found to be associated with the strong inhibitory effect of CDCA upon some ion transporters [Maléth et al., 2011]; (4) changes in intracellular phosphorylation/dephosphorylation mechanisms, as the amount/activity of many membrane transporters, including MCT1 [Alrefai et al., 2004; Narumi et al., 2010], is modulated by these mechanisms, and intracellular signaling mechanisms, such as PKC, MAPK, Ca^{2+}, and phosphoinositide-3 kinase have been identified as molecular targets for bile acids [Qiao et al., 2000; McMillan et al., 2003]; and (5) increased production of reactive oxygen species (ROS) and oxidative stress [Song et al., 2007; Ignacio Barrasa et al., 2011].

We decided to clarify the role of intracellular regulatory pathways in the regulation of ^{14}C-BT uptake by CDCA in IEC-6 cells, by examining the effect of inhibitors of various intracellular regulatory pathways, including pathways mediated by CaM, PTK, PKA, PKC, PKG, MAPK ERK1/2, and p38 MAPK. Our study revealed that the inhibitory effect of CDCA upon ^{14}C-BT uptake was partially reversed by inhibition of CaM, PKC, and the MAPK pathways ERK1/2 and p38. This observation suggests that CDCA-induced inhibition of ^{14}C-BT uptake is dependent on CaM, PKC, MAPK ERK1/2, and p38 activation. The stimulatory effect of CDCA upon MAPK is in agreement with previous reports obtained with some other bile acids [McMillan et al., 2003], and PKC is a known molecular target for bile acids [Qiao et al., 2000; McMillan et al., 2003].

We also decided to investigate the role of oxidative stress and ROS in the inhibitory effect of CDCA upon ^{14}C-BT uptake, because we recently demonstrated that oxidative stress decreases BT uptake in IEC-6 cells [Gonçalves et al., unpublished results], and proinflammatory cytokines, which inhibit SMCT1- and MCT1-mediated BT uptake [Thibault et al., 2007; Borthakur et al., 2010], also lead to the production of ROS [Seidelin and Nielsen, 2005; Babbar and Casero, 2006]. Increased production of ROS appears to be involved in the inhibitory effect of CDCA upon BT uptake, as NAC reduced the inhibitory effect of CDCA upon ^{14}C-BT uptake. This observation is of great importance, because oxidative stress is associated with the process of initiation and progression of colon carcinogenesis and inflammatory bowel disease [Seril et al., 2003; Almenier et al., 2012].

The ability of BT to modulate gene expression is often attributed to histone hyperacetylation through inhibition of histone deacetylases (HDACi) [Hamer et al., 2008]. Because these effects are dependent on the intracellular concentration of BT, it is expected that inhibition of BT cellular uptake will change its cellular effects. So, in the last part of this work, we evaluated the effect of BT in conjunction with CDCA upon viability, proliferation, and differentiation of IEC-6 cells.

BT (5 mM; 2 days) caused a significant reduction in IEC-6 cell proliferation and viability and a very marked increase on cell differentiation. This observation is in perfect agreement with our recent report obtained in this same cell line [Gonçalves et al., 2011c], and with the known effect of HDACi such as BT upon these parameters [Hamer et al., 2008]. CDCA (100 μM; 2 days) did not affect cell viability and differentiation, and caused only a discrete increase in IEC-6 proliferation. Interestingly enough, combination of CDCA with BT caused a reduction in the inhibitory effect of BT upon cell proliferation and differentiation. Unexpectedly, CDCA potentiated the effect of BT upon cell viability, a result for which we have at present no explanation.

In summary, we demonstrate that CDCA is an effective inhibitor of ^{14}C-BT uptake in both tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cells. In IEC-6 cells: (1) CDCA inhibits ^{14}C-BT uptake in a concentration-dependent manner, acting as a competitive inhibitor of the high-affinity transporter of ^{14}C-BT; (2) CDCA decreases both MCT1- and SMCT1-mediated uptake of ^{14}C-BT (although the inhibitory effect upon SMCT1 appears more pronounced); (3) CDCA induces an increase in SMCT1 mRNA steady-state levels; (4) ^{14}C-BT uptake is regulated by CaM, CaMKII, PKC, and PKA; (5) inhibition of ^{14}C-BT uptake by CDCA is dependent on CaM, MAPK ERK1/2, and p38 and PKC activation; and (6) CDCA significantly reduces the effects of BT upon cell proliferation and differentiation.

Given the very important homeostatic role played by BT at the intestinal epithelial level, knowledge on the interaction between an endogenous compound present in high amounts at that level (in cecum, 7–20% of total bile acids are CDCA [Hamilton et al., 2007] and in colon high concentrations are also present) and BT uptake into epithelial cells is very important. Indeed, the results of this study are very interesting in the context of the well-known anticarcinogenic role played by BT in the intestinal epithelium, as they suggest that inhibition of BT uptake into the intestinal epithelium might contribute to the procarcinogenic effect of CDCA at this level.

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VIII - The effect of oxidative stress on the intestinal epithelial uptake of butyrate

The effect of oxidative stress upon the intestinal epithelial uptake of butyrate

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ABSTRACT

Our aim was to investigate the effect of oxidative stress upon butyrate (BT) uptake at the intestinal epithelial level. For this, IEC-6 cells were treated with tert-butylhydroperoxide 3000 µM (tBOOH), which increased levels of oxidative stress biomarkers, while maintaining cellular viability. The effect of tBOOH upon uptake of 14C-BT (10 µM) can be summarized as follows: (a) it caused a reduction in the intracellular accumulation of 14C-BT over time, (b) it strongly reduced total 14C-BT uptake but did not affect Na+-independent uptake of 14C-BT, and (c) it did not affect the kinetics of 14C-BT uptake at 37°C, but increased uptake at 4°C. Moreover, tBOOH increased the efflux of 14C-BT not mediated by breast cancer resistance protein. We thus conclude that tBOOH strongly inhibits Na+-coupled monocarboxylate cotransporter 1 (SMCT1)-mediated, but not H+-coupled monocarboxylate transporter (MCT1)-mediated BT uptake; moreover, it increases uptake and efflux of BT by passive diffusion. tBOOH did not affect the mRNA expression levels of MCT1 and SMCT1 nor their cell membrane insertion. Rather, its effect was dependent on extracellular signal regulated kinase 1/2 (ERK 1/2) and protein tyrosine kinase (PTK) activation and on the generation of ROS by NADPH and xanthine oxidases and was partially prevented by the polyphenols quercetin and resveratrol. In conclusion, tBOOH is an effective inhibitor of SMCT1-mediated BT transport in non-tumoral intestinal epithelial cells. Given the important role played by BT in the intestine, this mechanism may contribute to the procarcinogenic and proinflammatory effect of oxidative stress at this level.

Keywords:

IEC-6 cells; tert-butylhydroperoxide; butyrate uptake; SMCT1; polyphenols

List of abbreviations:

BCRP- BCRP- breast cancer resistance protein; BT- butyrate; ERK 1/2- extracellular signal regulated kinase 1/2; GK - glucose-Krebs; GPx - total glutathione; GSH – reduced glutathione; GSSG - oxidized glutathione; LDH - lactate dehydrogenase; MAPK- mitogen-activated protein kinase; MCT1- monocarboxylate transporter 1; MTT- 3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide; PTK- protein tyrosine kinases; QRT-PCR – real-time quantitative reverse transcription polymerase chain reaction; ROS- reactive oxygen species; SMCT1- sodium-coupled monocarboxylate cotransporter 1; SRB- sulforhodamine B; TBARS - thiobarbituric acid reactive substances; tBOOH – tert-butylhydroperoxide

1. Introduction

Reactive oxygen species (ROS) are naturally produced as a result of oxygen metabolism. Under physiological conditions, the burden of ROS production is largely neutralized by an intricate antioxidant defense system (Wojcik et al., 2010). Increased ROS level, also known as oxidative stress, is a result of either increased ROS generation and/or a loss of antioxidant defense mechanisms (Khandrika et al., 2009). A major consequence of oxidative stress is damage of tissue via direct oxidation of nucleic acid bases, lipids and proteins, but also via profound alterations in signal transduction pathways, which can severely compromise cellular functions (Okayama, 2005). Not surprisingly, it is associated with numerous pathologies, from atherosclerosis to inflammation and cancer (Halliwell, 2001; Klaunig and Kamendulis, 2004; Stocker and Keaney, 2004).

The gastrointestinal tract is a major target for oxidative stress damage due to constant exposure of ROS generated by a large variety of xenobiotics, endogenous toxic substances (e.g. bile acids), as well as microbes and their products (Ames, 1983). Interestingly, the etiology of many gastrointestinal tract diseases, such as colon cancer or inflammatory bowel disease, is associated with an imbalance in the cellular redox system leading to increased levels of ROS (Acharya et al., 2010; Almenier et al., 2012; Seril et al., 2003). The effect of oxidative stress on membrane transport mechanisms at the intestinal level remains, however, poorly understood.

Butyrate (BT) a product of intestinal flora fermentation of dietary fibre, plays a key role in colonic epithelium homeostasis, by having multiple regulatory roles at that level, including: being the main energy source for colonocytes; promotion of growth and proliferation of normal colonic epithelial cells; inhibition of colon carcinogenesis; inhibition of colon inflammation; and inhibition of oxidative stress (Hamer et al., 2008; Wong et al., 2006). BT is transported into colonic epithelial cells by two specific carrier-mediated transport systems, the electroneutral H^+-coupled monocarboxylate transporter 1 (MCT1) and the Na^+-coupled monocarboxylate cotransporter (SMCT1) (Gupta et al., 2006). MCT1 (Cuff et al., 2005) and SMCT1 (Gupta et al., 2006) were recently proposed to function as tumor suppressors, most probably due to their ability to mediate the entry of BT into colonic epithelial cells. Therefore, factors that interfere with BT uptake into colonic epithelial cells are potentially detrimental to intestinal health and integrity by promoting oxidative stress, inflammation and colorectal cancer (Hamer et al., 2008; Wong et al., 2006).

Several studies have demonstrated that ROS can interfere with protein, including membrane transporters, activity (Akram et al., 2006; Kumar et al., 2007). However, nothing is known concerning the effect of oxidative stress upon the intestinal absorption of BT. So, the aim of this work was to investigate the effect of oxidative stress on ^{14}C-BT uptake by IEC-6 cells. IEC-6 cells are a nontumoral rat intestinal epithelial cell line (Wood et al., 2003). Oxidative stress was generated with *tert*-butylhydroperoxide (tBOOH), a useful model compound to study mechanisms of oxidative stress injury (Deiana et al., 2010; Garcia-Cohen et al., 2000; Griendling et al., 2000; Tupe and Agte, 2010).

2. Materials and Methods

2.1. IEC-6 and Caco-2 cell culture

The IEC-6 and Caco-2 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passages numbers 18-37 (IEC-6 cells) and 40-43 (Caco-2 cells). The cells were maintained in a humidified atmosphere of 5% CO_2-95% air. IEC-6 cells were cultured in Dulbecco's Modified Eagle's Medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma, St. Louis, MI, USA). Caco-2 cells were cultured in Minimum Essential Medium containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Sigma). Culture medium was changed every 2-3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21-cm^2; ∅ 60 mm; Corning Costar, Corning, NY, USA). For determination of cell viability, measurement of glutathione levels and ^{14}C-BT uptake studies, cells were seeded on 24-well plastic cell culture clusters (1.9 cm^2; Ø 15.4 mm; TPP®, Trasadingen, Switzerland), and the experiments were performed 8-9 days after the initial seeding. For measurement of lipid peroxidation (TBARS assay) and protein carbonyl groups, cells were seeded on 12-well plastic cells culture clusters (3.9 cm^2; Ø 21.4 mm; TPP®) and the experiments were performed 8-9 days after the initial seeding.

2.2. Treatment of cells with tert-butylhydroperoxide (tBOOH)

Before each experiment, the cell culture medium was removed and the wells were washed with Glucose-Krebs buffer at 37°C, containing in mM: 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4, 5.5 glucose and 20 HEPES, pH 7.4 (GK-HEPES buffer). IEC-6 or Caco-2 cells were then incubated for 1h at 37ºC with tBOOH (100, 1000 or 3000 µM) in GK-HEPES buffer. The effect of antioxidants and inhibitors of intracellular signalling pathways was tested by incubating IEC-6 cells in GK-HEPES buffer containing these compounds (or the respective solvents) for 20 min followed by incubation with tBOOH 3000 µM for 1h in the presence of these compounds (or the respective solvents).

2.3. Evaluation of tBOOH-induced oxidative stress

The magnitude of oxidative stress induced by tBOOH was indirectly evaluated, by measuring total (GSx), oxidized (GSSG) and reduced (GSH) glutathione levels and generation of lipid peroxidation products and protein carbonyl groups.

2.3.1 Measurement of total (GSx), oxidized (GSSG) and reduced (GSH) glutathione levels

IEC-6 cells were seeded on 24-well plates and submitted to treatment with tBOOH. Measurement of intracellular GSx levels was carried out according to a previously published method

(Capela et al., 2007). Briefly, cultured cells were scraped and proteins precipitated with perchloric acid 5%, then centrifuged for 10 min at 4ºC and the supernatant was neutralized with an equimolar solution of $KHCO_3$. GSx content was measured by the rate of colorimetric change of 0.7 mM 5,5-dithiobis(nitrobenzoic acid) at 415 nm in the presence of 0.4 U of GSH redutase and 0.24 mM NADPH, using a microplate reader. GSSG was also quantified, using 2-vinylpiridine to block free SH groups. GSH levels were calculated according to the following reaction: GSx = GSH + 2 GSSG.

2.3.2. Measurement of lipid peroxidation products (TBARS assay)

IEC-6 cells were seeded on 12-well plates and submitted to treatment with tBOOH. The extent of lipid peroxidation, which can be determined as the formation of malondialdehyde after the breakdown of polyunsaturated fatty acids, was measured by the thiobarbituric acid reactive substances (TBARS) assay (Fernandes et al., 1995). Briefly, 300 µl of cell suspension was precipitated with 200 µl of 50% trichloroacetic acid (TCA) and centrifuged for 1 min at 6000 rpm. 300 µl of the supernatant were added to an equal volume of 1% thiobarbituric acid and the mixture was heated for 40 minutes at 95ºC, allowed to cool and the absorvance measured at 535 nm.

2.3.3. Measurement of protein carbonyl groups

IEC-6 cells were seeded on 12-well plates and submitted to treatment with tBOOH. Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. Protein carbonyl content is the most used marker of protein oxidation (Dalle-Donne et al., 2003). The detection of protein carbonyl groups involves their reaction with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product, followed by the spectrophotometric quantification of the acid hydrazones (Levine et al., 1990). Briefly, carbonyl content was measured in the resultant pellet, that was treated with 0.5 ml of 2,4-dinitrophenylhydrazine (10 mM in HCl 2 M) or 0.5 ml of HCl 2 M for the blank. Samples were incubated for 1h at room temperature, vortexing every 10 min. 0.5 ml of TCA 20% was added to each tube which was allowed to stand for 15 min at 4°C. The resultant pellet was washed 3 times with ethanol-ethyl acetate (1:1), centrifuged at 13000 rpm for 2 min at 4°C and dissolved in 1 ml guanidine 6 M overnight. The solution was then centrifuged at 3000 rpm for 15 min. Absorbance was read at 340 nm and carbonyl content was calculated using the extinction coefficient of 22 000 M^{-1} cm^{-1}.

2.4. Evaluation of tBOOH effect on cell viability

2.4.1. Quantification of extracellular LDH activity

IEC-6 cells were seeded on 24-well plates and submitted to treatment with tBOOH (100, 1000 and 3000 µM). Cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular medium was measured spectrophotometrically by measuring the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described by Bergmeyer and Bernt (Bergmeyer, 1974).

2.4.2. Sulforhodamine B (SRB) assay

IEC-6 cells were seeded on 24-well plates and submitted to treatment with tBOOH (100, 1000 and 3000 µM). After treatment, 62.5 µl of ice-cold 50% (w/v) TCA were added to the culture medium on each well to fix cells (1h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed and cultures were rinsed four times with 1% (v/v) acetic acid to remove residual dye. Plates were again air-dried and the bound dye was then solubilized with 375 µl of 10 mM Tris.NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm (Goncalves et al., 2011b).

2.4.3. MTT (3-{4,5-dimethyl-2-thiazolyl}-2,5-diphenyl-2H-tetrazolium bromide) assay

IEC-6 cells were seeded on 24-well plates and submitted to treatment with tBOOH (1000 and 3000 µM). After treatment, 50 µl MTT solution (5 mg/ml) were added to each well. The cells were then further incubated for 3 h at 37 ºC. The formazan crystals derived from MTT cleavage were then measured as described by (Mosmann, 1983).

2.5. Determination of ^{14}C-BT uptake

Uptake experiments were performed with IEC-6 or Caco-2 cells incubated in GK-MES buffer (GK buffer in which HEPES is substituted by an equimolar concentration of MES), pH 6.5 (except in pH-dependence experiments), after treatment with tBOOH. In the experiments of Na^+ dependence, NaCl was substituted by an equimolar concentration of LiCl.

Initially, the buffer was aspirated and the cells were washed with 0.3 ml buffer GK-MES at 37ºC; then, the cell monolayers were preincubated for 20 min in 0.3 ml GK-MES buffer at 37ºC. Uptake of ^{14}C-BT was initiated by addition of 0.3 ml GK-MES buffer at 37ºC containing ^{14}C-BT 10 µM (except in kinetic experiments). At the end of the incubation period (3 min, except in time-course experiments), incubation was stopped by placing the cells on ice and rinsing the cells with 0.3 ml ice-cold GK-MES buffer. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4), and placed at 37ºC overnight. Radioactivity in the cells was measured by liquid scintillation counting.

2.6. Determination of ^{14}C-BT efflux

Initially, the culture medium was aspirated, and the cells were washed twice with 0.3 ml of GK-MES buffer at 37°C. Then the cell monolayers were preincubated for 1h with 0.3 ml of GK-MES buffer at 37°C, and uptake was initiated by the addition of 0.3 ml of GK-MES buffer at 37°C containing ^{14}C-BT 10 µM. Incubation was stopped after 30 min by removing the incubation buffer and rinsing the cells with 0.5 ml of ice-cold buffer. Then, efflux was measured by incubating the cells with 0.3 ml of GK-MES buffer at 37°C for 20 min. At the end of this period, the medium was collected, and the cells were solubilized with 0.3 ml of 0.1% (v/v) Triton X-100 (in 5 mM Tris·HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in both the efflux buffer and the cells was measured by liquid scintillation counting. Compounds to be tested were present during the efflux period only.

2.7. Reverse transcription quantitative real-time-PCR (qRT-PCR)

Total RNA was extracted from IEC-6 cells using the Tripure® isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Germany). Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen Corporation, CA, USA) according to manufacturer's instructions, and 10 µg of resulting DNA-free RNA were reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 40 µl of final reaction volume, according to the manufacturer's instructions. Resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade unreacted RNA. For the quantitative real-time PCR, 2 µl of the 40 µl reverse transcription reaction mixture were used. For the calibration curve, IEC-6 standard cDNA was diluted in five different concentrations.

Real-time PCR was carried out using a LightCycler (Roche, Nutley, NJ, USA). 20 µl reactions were set up in microcapillary tubes using 0.5 µM of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). Cycling conditions were as follows: denaturation (95ºC for 5 min), amplification and quantification (95ºC for 10 s, annealing temperature (AT) for 15 s, and 72ºC for 10 s, with a single fluorescence measurement at the end of the 72ºC for 10 s segment) repeated 50 times, a melting curve program ((AT + 10) ºC for 15 s and 95ºC with a heating rate of 0.1ºC/s and continuous fluorescence measurement), and a cooling step to 40ºC (30 s). Annealing temperatures (AT) and sequence of primers are indicated in Table 1. Data were analyzed using LightCycler® 4.05 analysis software (Roche, Mannheim, Germany).

TABLE I. Primers Used in qRT-PCR

Gene name	Primer sequence (5′–3′)	AT (°C)
rGAPDH	F: GGC ATC GTG GAA GGG CTC ATG AC R: ATG CCA GTG AGC TTC CCG TTC AGC	62
rMCT1	F: CAG TGC AAC GAC CAG TGA ATG TG R: ATC AAG CCA CAG CCA GAC AGG	69
rSMCT1	F: CGG GAT CAC CAG CAC CTA C R: GCA GGG GCA TAA ATC ACA ATC	62

rGAPDH, rat glyceraldehyde-3-phosphate dehydrogenase; rMCT1, rat monocarboxylate transporter type 1; rSMCT1, rat Na^+-coupled monocarboxylate transporter type 1; F, forward; R, reverse.

2.8. Protein determination

The protein content of cell monolayers was determined as described by Bradford (Bradford, 1976) using serum albumin as standard.

2.9. Calculation and statistics

For the analysis of the time course of ^{14}C-BT uptake, the parameters of Eq.1 were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method (Muzyka A, 2005).

$$A(t) = k_{in} / k_{out}(1 - e^{-k_{out} \times t}) \quad (1)$$

A(t) represents the accumulation of ^{14}C-BT at time, k_{in} and k_{out} are the rate constants for inward and outward transport, respectively, and *t* is the incubation time. A_{max} corresponds to the accumulation (*A(t)*) at steady state ($t \rightarrow \infty$). k_{in} is given in nmol per milligram protein per min and k_{out} in min^{-1}. In order to obtain clearance values, k_{in} was converted to µl per milligram protein per min.

For the analysis of the saturation curve of ^{14}C-BT uptake, the parameters of the Michaelis-Menten equation were fitted to the experimental data (Muzyka A, 2005).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. Statistical analysis of the difference between various groups was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. Statistical analysis of the difference between two groups was evaluated with Student's t test. Differences were considered to be significant when $P<0.05$.

2.10. Materials

^{14}C-BT ([1-^{14}C]-*n*-butyric acid, sodium salt; specific activity 30-60 mCi/mmol) (Biotrend Chemikalien GmbH, Köln, Germany); DMSO (dimethylsulfoxide), (−)-cis-3,3′,4′,5,5′,7-hexahydroxy-flavane-3-gallate ((−)-epigallocatechin-3-gallate), (E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile (Bay 11-7082), ′5,7-trihydroxyisoflavone (genistein), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY-294,002), 3,3′,4′,5,6-pentahydroxyflavone (quercetin), 3,4′,5-trihydroxy-trans-stilbene (resveratrol), 4′,5,7-trihydroxyisoflavone 7-glucoside (genistin), 5,5-dithiobis(nitrobenzoic) acid, 2,4-dinitrophenylhydrazine (DNP), acetic acid sodium salt, calmidazolium, chelerythrine chloride, colchicine, H-89 dihydrochloride hydrate, KN-62, decane, ethanol, GSH redutase, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), (2-[N-morpholino] ethanesulfonic acid hydrate) (MES), Minimum Essential Medium, monensin sodium salt, nicotinamide adenine dinucleotide (NADH), β-nicotinamide adenine dinucleotide phosphate (NADPH), PD 98058, penicillin/streptomycin/amphotericin B solution, pyruvic acid sodium salt, rapamycin, SB 203580, serum albumin, SP 600125, sodium pyruvate, sulforhodamine, *tert*-butylhydroperoxide (tBOOH), thiobarbituric acid, trichloroacetic acid sodium salt, Tris-HCl, Tris.NaOH, trypsin–EDTA solution, 2-vinylpiridine (Sigma, St. Louis, MO, USA); perchloric acid, triton X-100 (Merck, Darmstadt, Germany).

Compounds to be tested were dissolved in H_2O, decane, $NaHCO_3$ (100 mM) dimethylsulfoxide or ethanol. The final concentration of these solvents in the buffer was 1%. Controls for these compounds were run in the presence of the respective solvent.

3. Results

3.1. Evaluation of tBOOH-induced oxidative stress in IEC-6 cells

IEC-6 cells were exposed to increasing concentrations of tBOOH, as described under Methods. Proposed mechanisms of tBOOH-induced toxicity include production of *tert*-butoxyl, peroxyl, alkoxyl and methyl radicals that catalyze lipid peroxidation, production of DNA stand breaks and alteration in intracellular calcium homeostasis following glutathione and protein thiol depletion (Aherne and O'Brien, 2000; Chamulitrat, 1998).

In order to assess the effect of tBOOH on IEC-6 cell oxidative stress levels, the cellular levels of total (GSx), oxidized (GSSG) and reduced (GSH) glutathione and GSH/GSSG ratio were determined. Treatment of IEC-6 cells with tBOOH 1000 and 3000 µM for 1h induced a significant decrease in both GSx and GSH cellular levels, although GSSG levels were not significantly affected; consequently, a marked decrease in the GSH/GSSG ratio was observed (Fig. 1).

To further evaluate the effect of tBOOH upon oxidative stress levels, we then measured the extent of lipid peroxidation, by using the TBARS assay (lipid peroxidation results in the formation of reactive aldehydes, including malondialdehyde), and the extent of protein carbonylation, by measuring protein carbonyl groups (produced on protein side chains when they are oxidized). tBOOH 3000 µM significant increased the cellular concentration of malondialdehyde (MDA), indicating oxidative damage to cell lipids (Fig. 2A), and tBOOH 1000 and 3000 µM significant increased the cellular concentration of carbonyl groups, indicating oxidative damage to proteins (Fig. 2B).

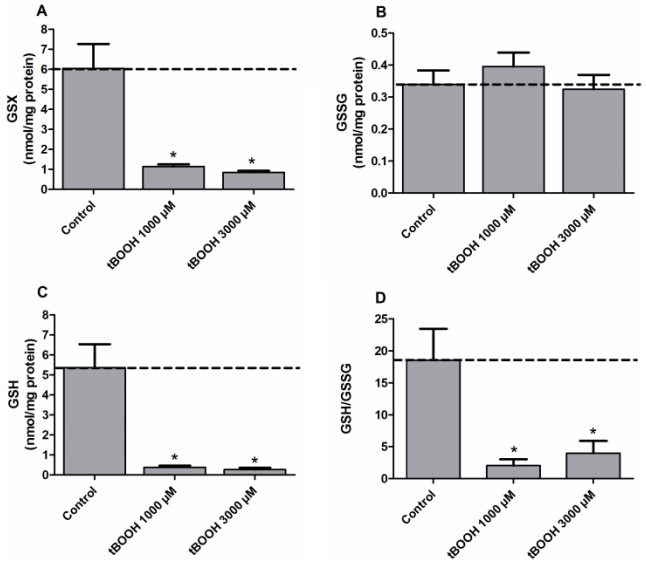

Figure 1. Effect of 1h-exposure to increasing concentrations of tBOOH (1000 and 3000 µM) on IEC-6 total glutathione (GSx) levels (A), oxidized glutathione (GSSG) levels (B), reduced glutathione (GSH) levels (C) and GSH/GSSG ratio (D). Results are expressed as nmol/mg protein (A, B and C). Shown are arithmetic means±SEM (n=17-20). Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. *Significantly different from control ($P<0.05$).

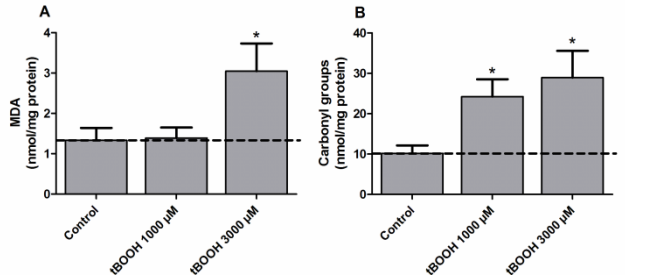

Figure 2. Effect of 1h-exposure to increasing concentrations of tBOOH (1000 and 3000 µM) on IEC-6 lipid peroxidation (A) and protein carbonyl (B) levels. (A) Lipid peroxidation was determined by quantification of MDA, as described in Methods. Results are shown as nmol MDA /mg protein (n=18-20). (B) Protein carbonil groups were determined as described in Methods. Results are shown as nmol/mg protein (n=14). Shown are arithmetic means±SEM. Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. *Significantly different from control ($P<0.05$).

3.2. Effect of tBOOH on IEC-6 cell viability

Next, we evaluated the effect of tBOOH upon IEC-6 cellular viability. Exposure of IEC-6 cells to increasing concentrations of tBOOH (100, 1000 and 3000 µM) caused no increase in LDH leakage, indicating that these concentrations did not affect cell membrane integrity (Fig. 3A). Similarly, treatment with tBOOH (100, 1000 and 3000 µM) was devoid of effect upon IEC-6 whole-cell protein (SRB assay) (Fig. 3B). Finally, tBOOH (1000 and 3000 µM) did not affect MTT cleavage (Fig. 3C), indicating that these concentrations did not affect the mitochondrial metabolic capacity of IEC-6 cells. Altogether, these results indicate that treatment of IEC-6 cells for 1h with tBOOH 3000 µM induced a significant increase in ROS formation, as indicated by an enhancement in lipid peroxidation, protein carbonylation and by a decrease in GSx and GSH levels and in GSH/GSSG ratio, while having no impact on cell viability. In other studies, tBOOH was also used as oxidative stress inducer at similar concentrations (Couto MR, 2012; Deiana et al., 2010). So, tBOOH 3000 µM was used in subsequent experiments aimed at determining the effect of oxidative stress upon ^{14}C-BT uptake by IEC-6 cells.

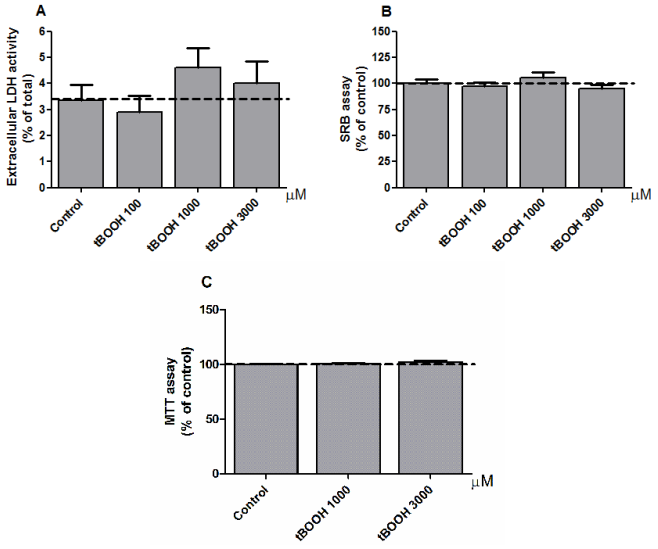

Figure 3. Effect of 1h-exposure to increasing concentrations of tBOOH (100, 1000, 3000 µM) on IEC-6 cell viability. (**A**) Quantification of extracellular LDH activity was done as described in Methods. Results are shown as extracellular LDH activity (% of total) (n=11-12). (**B**) Whole cellular protein was quantified with the SRB assay as described in Methods. Results are shown % of control (n=10-12). (**C**) The MTT assay was done as described in Methods. Results are shown as % of control (n=10). Shown are arithmetic means±SEM. Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test.

3.3. Effect of tBOOH upon ^{14}C-BT uptake

3.3.1. Effect upon the time-course of ^{14}C-BT uptake in IEC-6 cells

In a first series of experiments, we determined the time-course of ^{14}C-BT (10 µM) accumulation in IEC-6 cells. For this, cells were incubated at 37ºC with ^{14}C-BT for various periods of time, in the absence or presence of a previous treatment with 3000 µM

tBOOH for 1h. As shown in Fig. 4A, treatment with tBOOH originated a reduced accumulation of ^{14}C-BT over time. Analysis of the time-course of ^{14}C-BT accumulation showed a marked decrease in the A_{max} in the presence of tBOOH (2.85±0.30 and 0.36±0.03 nmol/mg prot in control and tBOOH-treated cells, respectively).

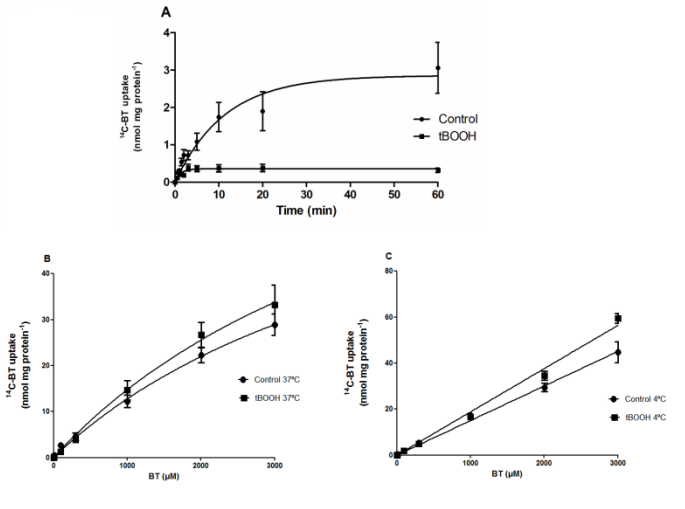

Figure 4. Time-course (**A**) and kinetic parameters (**B, C**) of ^{14}C-BT uptake by IEC-6 cells, after treatment for 1h with tBOOH 3000 µM (tBOOH) or the respective solvent (Control). (**A**) Cells were incubated at 37ºC with 10 µM ^{14}C-BT for different periods of time (n=5-7). (**B**) Initial rates of ^{14}C-BT uptake were determined in cells incubated at 37°C with 0.01–3 mM of ^{14}C-BT for 3 min (n=6). (**C**) Initial rates of ^{14}C-BT uptake were determined in cells incubated at 4°C with 0.01–3 mM of ^{14}C-BT for 3 min (n=6-8). Shown are arithmetic means±SEM. Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test.

3.3.2. Effect upon the kinetics of ^{14}C-BT uptake in IEC-6 cells

The time-course results (section 3.3.1.) and previous results from our group (Goncalves et al., 2011a) show that the apical uptake of ^{14}C-BT in IEC-6 cells is linear with time for up to 3 min of incubation. So, the kinetics of ^{14}C-BT uptake by IEC-6 cells was next determined, by measuring initial rates of ^{14}C-BT uptake at increasing substrate concentrations (10-3000 µM) at 37ºC for 3 min. We verified that tBOOH 3000 µM was devoid of significant effect upon the kinetics of ^{14}C-BT uptake (V_{max}=78.5±22.5 and 94.4±40.4 nmol mg prot^{-1} 3 min^{-1}; K_m=5.14±2.14 and 5.38±3.29 mM, in control and tBOOH-treated cells, respectively). However, analysis of the saturation curve reveals that tBOOH decreased uptake of low concentrations and increased uptake of high concentrations of ^{14}C-BT (Fig. 4B).

At 37ºC both carrier and non-carrier mediated mechanisms of transport are functional. To distinguish between the two routes of transport, we also performed saturation curves at 4ºC. In this condition, only the non-carrier mediated transport is present. As shown in Fig. 4C, uptake of ^{14}C-BT at 4°C was linear with increasing concentrations of ^{14}C-BT, and treatment with tBOOH increased the slope of ^{14}C-BT uptake, compared to control (0.01884±0.00054 and 0.01505±0.00027 for control and tBOOH-treated cells, respectively). So, tBOOH increased passive diffusional uptake of ^{14}C-BT. However, it should be noted that this was found only for high concentrations of BT (2 mM and above); for the concentration of ^{14}C-BT used in most of the experiments of this study (10 µM), passive diffusional transport has a minor importance.

3.3.3. Effect upon MCT1- and SMCT1-mediated ^{14}C-BT uptake

Uptake of ^{14}C-BT by IEC-6 cells involves both MCT1- and SMCT1- mediated transport (Goncalves et al., 2011a). To distinguish between the effect of tBOOH upon MCT1-mediated (extracellular Na^+-independent) and SMCT1-mediated (extracellular Na^+-dependent) uptake, we compared the inhibitory effect of tBOOH on ^{14}C-BT accumulation in the presence (total uptake) and absence of NaCl (Na^+-independent uptake), at different pHs (Fig. 5).

In agreement with a previous publication from our group (Goncalves et al., 2011a), ^{14}C-BT uptake by IEC-6 cells involves both a Na^+-dependent and a Na^+-independent component (Figs. 5A and 5B). In both control and tBOOH-treated cells, total and Na^+-independent ^{14}C-BT uptake was strongly pH-dependent, being significantly increased with a decrease in pH (Figs. 5A and 5B). Interestingly enough, tBOOH strongly inhibited total ^{14}C-BT uptake (MCT1 and SMCT1-mediated uptake), but did not inhibit (and even increased, at pH 7.5) Na^+-independent (MCT1-mediated) uptake of ^{14}C-BT. We can thus conclude that tBOOH inhibits SMCT1-mediated uptake of ^{14}C-BT in IEC-6 cells, but does not affect MCT1-mediated uptake. In order to confirm this conclusion, we decided to investigate the effect of tBOOH in Caco-2 cells, in which BT uptake is mainly MCT1-mediated (Goncalves et al., 2009). Our group had previously shown that the apical uptake of ^{14}C-BT in Caco-2 cells was linear with time for up to 3 min of incubation (Goncalves et al., 2009) and that 3000 µM tBOOH for 1h induced oxidative stress (Couto MR, 2012). As shown in Fig. 5C, tBOOH was devoid of effect upon ^{14}C-BT uptake in Caco-2 cells, supporting the conclusion that oxidative stress does not affect MCT1-mediated transport.

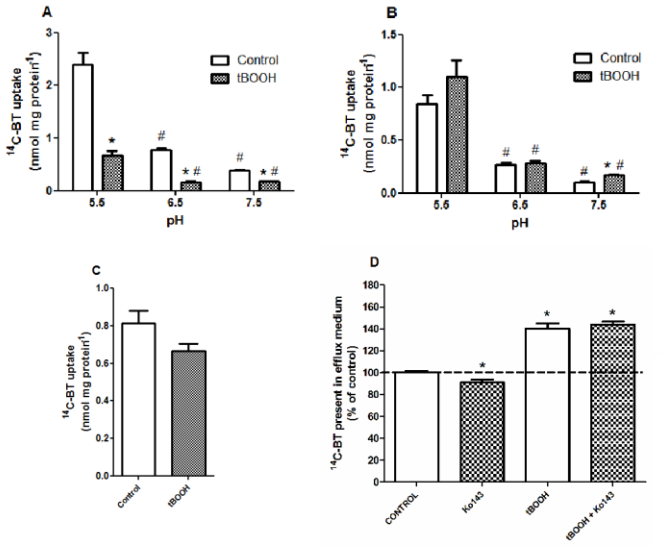

Figure 5. (**A**) Effect of tBOOH on ^{14}C-BT uptake by IEC-6 cells in the presence of extracellular Na^+ (total uptake). (**B**) Effect of tBOOH on ^{14}C-BT uptake by IEC-6 cells in the absence of extracellular Na^+ (Na^+-independent uptake). (**C**) Effect of tBOOH on ^{14}C-BT uptake by Caco-2 cells. (**D**) Effect of tBOOH on ^{14}C-BT efflux from IEC-6 cells. (**A**) Cells were incubated at 37ºC for 3 min with ^{14}C-BT (10 µM) in GK-MES (pH 5.5 and 6.5) or GK-HEPES (pH 7.5) buffer, in the absence (control) or presence of a previous exposure to tBOOH 3000 µM (tBOOH) for 1h (n=9-12). (**B**) Cells were incubated at 37ºC for 3 min with ^{14}C-BT (10 µM) in GK-MES (pH 5.5 and 6.5) or GK-HEPES (pH 7.5) buffer where NaCl was isotonically replaced by LiCl, in the absence (control) or presence of a previous exposure to tBOOH 3000 µM (tBOOH) for 1h (*n*=6-12). (**C**) Initial rates of ^{14}C-BT uptake were determined in Caco-2 cells incubated at 37°C with ^{14}C-BT (10 µM) for 3 min, after treatment for 1h with tBOOH 3000 µM (tBOOH) or the respective solvent (Control) (*n*=14-15). (**D**) Cells were incubated in GK-MES (pH 6.5) at 37ºC for 30 min with ^{14}C-BT (10 µM), and then efflux of ^{14}C-BT from the cells was measured for 20 min, in the absence or presence of tBOOH (3000 µM) or Ko143 1 µM (Ko143), as explained in the Methods (*n*=8-12). Results are shown as nmol/mg protein (arithmetic means±SEM). Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. *Significantly different from the respective control; #Significantly different from pH 5.5 ($P<0.05$).

3.3.4. Effect of tBOOH upon ^{14}C-BT efflux

The intracellular accumulation of ^{14}C-BT depends not only on uptake mechanisms, but is also dependent on efflux mechanisms, which are able to remove ^{14}C-BT from the cells. So, we decided to investigate also the effect of tBOOH upon ^{14}C-BT efflux from IEC-6 cells.

As shown in Fig. 5D, efflux of ^{14}C-BT from IEC-6 cells was strongly increased by tBOOH. BCRP was recently found to be involved in the efflux of ^{14}C-BT in IEC-6 cells (Goncalves et al., 2011b). In order to investigate if tBOOH affects BCRP-mediated ^{14}C-BT efflux, we tested the influence of a BCRP inhibitor (Ko143 1µM) (Allen et al., 2002). In agreement with the fact that ^{14}C-BT is effluxed out of the cells through BCRP, Ko143 decreased efflux of ^{14}C-BT in control cells. However, in the presence of tBOOH, efflux of ^{14}C-BT was not affected by Ko143, indicating that BCRP is not involved (Fig. 5D).

3.4. Effect of tBOOH upon MCT1 and SMCT1 mRNA levels in IEC-6 cells

A comparison between the mRNA steady-state levels of MCT1 and SMCT1 in control and tBOOH-treated IEC-6 cells, evaluated by qRT-PCR, shows that MCT1 and SMCT1 mRNA levels were similar in control and tBOOH-treated cells (Fig. 6).

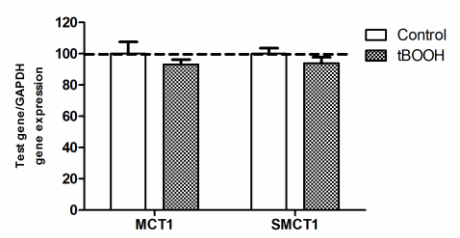

Figure 6. Quantification of mRNA levels of rat H^+-coupled monocarboxylate transporter 1 (MCT1) and rat Na^+-coupled monocarboxylate cotransporter (SMCT1), by qRT-PCR, in IEC-6 cells after treatment for 1h with tBOOH 3000 µM (tBOOH) or its solvent (Control; *n*=5). Results are shown as the expression of MCT1 or SMCT1 relative to GAPDH (arithmetic means±SEM). Statistical analysis was evaluated with Student's t test. * Significantly different from control ($P<0.05$).

3.5. Effect of modulators of intracellular signaling pathways on tBOOH-induced inhibition of ^{14}C-BT uptake in IEC-6 cells

Next, we investigated the signaling mechanisms that are involved in the inhibition of ^{14}C-BT uptake caused by tBOOH in IEC-6 cells. The signaling pathways investigated were chosen based on the fact that they are known to be activated by ROS: protein kinase C, mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase/Akt/mTOR pathway, nuclear factor-kappaB (NF-kB) signaling, and protein tyrosine kinases (Benhar et al., 2002; Liu et al., 2006; Poli et al., 2004; Wu, 2006).

First, because oxidative stress led to elevations of cytosolic Ca^{2+} concentration (Stone et al., 1994) and a synergistic action of Ca^{2+} is required for activation of classical Ca^{2+}-mediated protein kinase C (Nishizuka, 1986), we analyzed the effect of the Ca^{2+}/calmodulin inhibitor calmidazolium and of the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62 (Said et al., 1999) upon tBOOH-induced inhibition of ^{14}C-BT uptake. Although uptake of ^{14}C-BT was significantly reduced in the presence of these agents, suggesting that BT uptake is dependent on intracellular Ca^{2+}/calmodulin and Ca^{2+}/calmodulin-dependent

protein kinase II, the effect of tBOOH upon ^{14}C-BT uptake was not changed by any of them (Fig. 7A).

Next, we studied the effect of specific inhibitors of protein kinase C (chelerythrine) (Herbert et al., 1990) and protein kinase A (H-89) (Chijiwa et al., 1990). H89 was devoid of effect, but chelerythrine markedly reduced ^{14}C-BT uptake, which is in agreement with previous studies showing that ^{14}C-BT uptake is stimulated by protein kinase C (Alrefai et al., 2004; Gonçalves P., 2012). However, the effect of tBOOH was also not changed in the presence of these agents (Fig. 7A).

The involvement of mitogen-activated protein (MAP) kinases was studied by testing the effect of specific inhibitors of mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase 1/2 (ERK1/2) (PD 98059) (Dudley et al., 1995), p38 MAPK (SB 203580) (Cuenda et al., 1995) and c-Jun-NH_2-terminal kinases (JNK) (SP 600125) (Bennett et al., 2001). PD 98059, SB 203580 and SP 600125 were devoid of effect upon ^{14}C-BT uptake, and SB 203580 and SP 600125 also did not change the inhibitory effect of tBOOH upon ^{14}C-BT uptake. However, PD 98059 slightly decreased it, suggesting that inhibition of ^{14}C-BT uptake by tBOOH depends on MAPK ERK1/2 activation (Fig. 7A).

Next, the involvement of the phosphatidylinositol-3-kinase/Akt/mTOR pathway was studied by testing the effect of specific inhibitors of phosphatidylinositol-3-kinase (LY 294002) (Vlahos et al., 1994) and mTOR (rapamycin) (Brown et al., 1994). Rapamycin was devoid of effect upon ^{14}C-BT uptake, but LY 294002 reduced it by 10%, thus appearing that BT uptake is dependent on activation of this pathway. However, the effect of tBOOH was not changed in the presence of these agents (Fig. 7B).

The involvement of nuclear factor-kappa B (NFκB) signal transduction pathway was studied by testing the effect of BAY 11-7082, a specific inhibitor of IkBa phosphorylation and degradation (Pierce et al., 1997). BAY 11-7082 was devoid of effect upon ^{14}C-BT uptake and upon the inhibitory effect of tBOOH on this parameter (Fig. 7B).

Finally, we also tested for the involvement of protein tyrosine kinases (PTKs) in tBOOH-mediated inhibition of ^{14}C-BT uptake by using genistein, a known PTK inhibitor (Akiyama et al., 1987). As shown in Fig. 7B, genistein, but not its negative control genistin (which does not exerts inhibition of PTK) reduced ^{14}C-BT uptake and decreased the inhibitory effect of tBOOH upon ^{14}C-BT uptake, suggesting that both BT uptake and inhibition of ^{14}C-BT uptake caused by tBOOH are dependent on PTK activation.

Cytoskeletal elements, such as microtubules, actin filaments, and their associated molecular motors, are intimately involved in mechanical signaling (Janmey, 1998). Because oxidative stress induced internalization of the bile salt export pump (Perez et al., 2006) we also tested the involvement of this mechanism in the inhibitory effect of tBOOH upon ^{14}C-BT uptake, by testing the effect of a microtubule disruptor (colchicine) (Malawist.Se and Bensch, 1967). The observation that colchicine was devoid of effect upon ^{14}C-BT uptake and did not change the effect of tBOOH upon it, demonstrates that oxidative stress does not affect ^{14}C-BT uptake by substantially altering the cellular distribution of BT transporters (Fig. 7B).

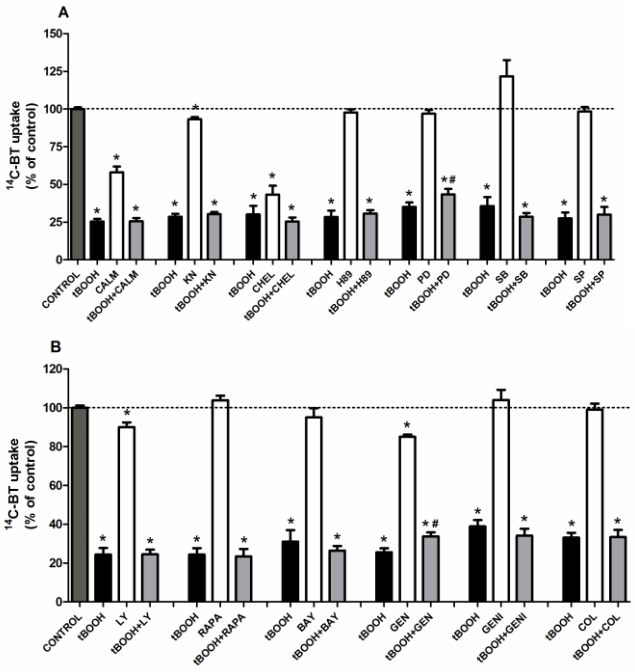

Figure 7. Influence of inhibitors of intracellular signalling pathways upon the inhibitory effect of tBOOH on ^{14}C-BT uptake by IEC-6 cells. (**A**) Cells were exposed to tBOOH 3000 µM (tBOOH), calmidazolium 25 µM (CALM), tBOOH+calmidazolium 25 µM (tBOOH+CALM), KN-62 10 µM (KN), tBOOH+KN-62 10 µM (tBOOH+KN), chelerythrine 10 µM (CHEL), tBOOH+chelerythrine 10 µM (tBOOH+CHEL), H-89 10 µM (H89), tBOOH+H-89 10 µM (tBOOH+H89), PD 98058 25 µM (PD), tBOOH+PD 98058 25 µM (tBOOH+PD), SB 203580 25 µM (SB), tBOOH+SB 203580 2.5 µM (tBOOH+SB), SP 600125 25 µM (SP), tBOOH+SP 600125 25 µM (tBOOH+SP), or the respective solvents, as described in Methods (*n*=7-22). (**B**) Cells were exposed to tBOOH 3000 µM (tBOOH), LY 294002 10 µM (LY), tBOOH+LY 294002 10 µM (tBOOH+LY), rapamycin 500 nM (RAPA), tBOOH+rapamycin 500 nM (tBOOH+RAPA), BAY 11-7082 5 µM (BAY), tBOOH+BAY 11-7082 5 µM (tBOOH+BAY), genistein 50 µM (GEN), tBOOH+genistein 50 µM (tBOOH+GEN), genistin 50 µM (GENI), tBOOH+genistin 50 µM (tBOOH+GENI), colchicine 10 µM (COL), tBOOH+colchicine 10 µM (tBOOH+COL) or the respective solvents, as described in Methods (*n*=6-12). Shown are arithmetic means±SEM. Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. * significantly different from the respective control ($P<0.05$) $^{\#}$ significantly different from tBOOH ($P<0.05$).

3.6. Effect of inhibitors of ROS generating enzymes on tBOOH-induced inhibition of ^{14}C-BT uptake in IEC-6 cells

ROS are produced via a variety of cellular oxidative metabolic processes, including NADPH oxidase, xanthine oxidase, arachidonic acid metabolism by cyclooxygenases and lipoxygenases, and the mitochondrial respiratory chain (Kim et al., 2008). We demonstrated that the inhibition of ^{14}C-BT uptake in IEC-6 cells by tBOOH was dependent on MAPK ERK1/2 activation. Previous studies indicate that ERK1/2 activation leads to an increase in NADPH oxidase (Cevik et al., 2008; Choudhary et al., 2011; Dewas et al., 2000; Seru et al., 2004), xanthine oxidase (Abdulnour et al., 2006) and cytosolic phospholipase A2 (cPLA2) (Askarova et al., 2011; Borowitz and Montgomery, 1989) activities and thus in ROS production. So, we investigated the involvement of ROS-generating enzymes in the inhibition of ^{14}C-BT uptake in response to tBOOH.

The NADPH oxidase inhibitor apocynin (Stolk et al., 1994) and the well known xanthine oxidase inhibitor allopurinol (Delgado et al., 1966) were devoid of effect upon ^{14}C-BT uptake but slightly decreased the inhibitory effect of tBOOH on ^{14}C-BT uptake (Fig. 8A), suggesting the involvement of NADPH oxidase and xanthine oxidase in the inhibition of ^{14}C-BT uptake by tBOOH.

Finally, the involvement of phospholipase A2 may be excluded because its inhibitor quinacrine (Winocour et al., 1981) had no effect upon ^{14}C-BT uptake and did not change the effect of tBOOH upon ^{14}C-BT uptake (Fig. 8A).

3.7. Effect of antioxidants on tBOOH-induced inhibition of ^{14}C-BT uptake and increase in lipid peroxidation in IEC-6 cells

Reducing oxidative stress levels with antioxidant therapy is one of the strategies of chemoprevention (Roessner et al., 2008). So, in the last part of this work, we investigated the effect of some antioxidants in the inhibitory effect of tBOOH upon ^{14}C-BT uptake. We tested the ROS scavenger N-acetyl-cysteine, vitamin C, and the polyphenols epigallocatechin-3-gallate, quercetin and resveratrol (Wojcik et al., 2010).

As shown in Fig. 8B, apart from epigallocatechin-3-gallate, all tested antioxidants significantly reduced ^{14}C-BT uptake. The inhibitory effect of quercetin and resveratrol was already described by our group (Goncalves et al., 2011a). Interestingly enough, the inhibitory effect of tBOOH upon ^{14}C-BT uptake was strongly reduced by quercetin and resveratrol, although N-acetyl-cysteine, vitamin C and epigallocatechin-3-gallate had no effect. The inhibitory effect of quercetin upon tBOOH-induced changes in ^{14}C-BT uptake correlated well with its inhibitory effect upon tBOOH-induced oxidative damage, as evaluated by quantification of lipid peroxidation. Indeed, although quercetin alone was devoid of effect in this parameter (97.7±21.9% of control, *n*=12), it completely abolished the increase in lipid peroxidation caused by tBOOH (from 431.5±42.6% to 105.4±28.4 of control in the absence and presence of quercetin, respectively; *n*=12).

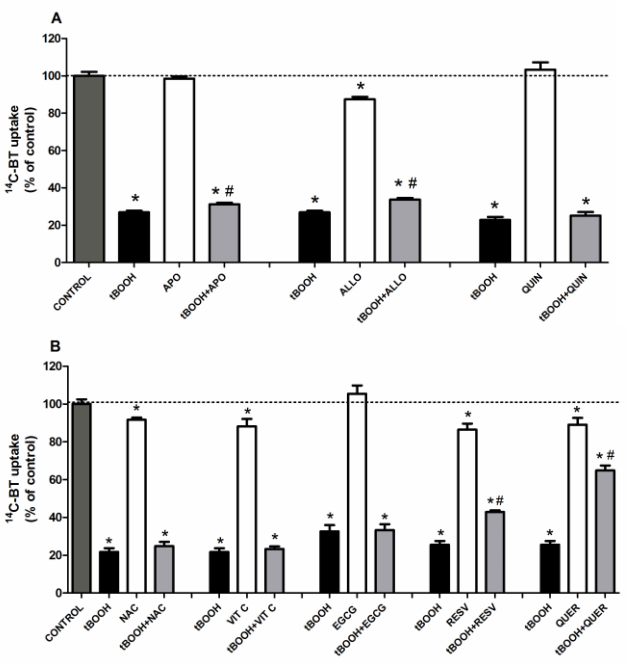

Figure 8. Influence of inhibitors of ROS generating enzymes (**A**) and antioxidants (**B**) upon the inhibitory effect of tBOOH on ^{14}C-BT uptake by IEC-6 cells. (**A**) Cells were exposed to tBOOH 3000 µM (tBOOH), apocynin 1 mM (APO), tBOOH+apocynin 1 mM (tBOOH+APO), allopurinol 1 mM (ALLO), tBOOH+allopurinol 1 mM (tBOOH+ALLO), quinacrine 5 µM (QUIN), tBOOH+quinacrine 5 µM (tBOOH+QUIN), or the respective solvents, as described in Methods (*n*=12). (**B**) Cells were exposed to tBOOH 3000 µM (tBOOH), N-acetyl-cysteine 1 mM (NAC), tBOOH+N-acetyl-cysteine 1 mM (tBOOH+NAC), vitamin C 100 µM (VIT C), tBOOH+vitamin C 100 µM (tBOOH+VIT C), epigallocatechin-3-gallate 50 µM (EGCG), tBOOH+epigallocatechin-3-gallate 50 µM (tBOOH+EGCG), resveratrol 50 µM (RESV), tBOOH+resveratrol 50 µM (tBOOH+RESV), quercetin 50 µM (QUER), tBOOH+quercetin 50 µM (tBOOH+QUER), or the respective solvents, as described in Methods (n=9-10). Statistical analysis was evaluated by the ANOVA test, followed by the Student-Newman-Keuls test. Shown are arithmetic means±SEM. * significantly different from the respective control ($P<0.05$) $^{\#}$significantly different from tBOOH ($P<0.05$).

4. Discussion

Based in previous studies (see Introduction) we chose tBOOH as a model to investigate intestinal oxidative stress. To validate this

model in IEC-6 cells, we exposed the cells to increasing concentration of tBOOH for 1h and then quantified some biomarkers of oxidative stress, and investigated the effect of tBOOH upon cell viability. In IEC-6 cells treated with 1000 and 3000 μM tBOOH, a significant decrease in both GSx and GSH levels was observed. GSH is the major endogenous thiol antioxidant, having an extensive role in the preservation of cellular redox balance as well as in the detoxification of exogenous and endogenous compounds (Dickinson and Forman, 2002). Accordingly, the observed decrease in GSH level is a good indicator of oxidative stress both *in vitro* and *in vivo* (Rossi et al., 2006). Oxidative stress induces cellular injury through the production of free radicals of high reactivity, leading to membrane lipid peroxidation (Poli et al., 1987) and oxidation of critical thiol groups, causing formation of homo- and heteroproteins bound by disulfide bridges (Di Monte et al., 1984), thus altering protein structure and function (Ottaviano et al., 2008). So, we also measured the extent of lipid peroxidation (TBARS assay) and protein carbonylation in response to tBOOH. We found a significant increase in lipid peroxidation in cells treated with 3000 μM tBOOH, and a significant increase in protein carbonylation in cells treated with tBOOH 1000 and 3000 μM.

As a whole, our results indicate that tBOOH seems to induce oxidative stress in IEC-6 cells, particularly at 3000 μM. This concentration of tBOOH had no impact on IEC-6 cell viability. Based on these results, we could conclude that IEC-6 cells submitted to treatment with 3000 μM tBOOH for 1h constitute a good cellular model to study the effects of oxidative stress upon the intestinal absorption of BT. We thus chose tBOOH 3000 μM for subsequent experiments.

The effect of tBOOH upon uptake of a low concentration of ^{14}C-BT (10 μM) by IEC-6 cells can be summarized as follows: (a) tBOOH caused a reduction in the intracellular accumulation of ^{14}C-BT over time; (b) tBOOH strongly reduced total ^{14}C-BT uptake but did not affect (or even increased) Na^+-independent uptake of ^{14}C-BT, and (c) tBOOH did not affect the kinetics of ^{14}C-BT uptake at 37°C (although it decreased uptake of low concentrations and increased uptake of high concentrations of ^{14}C-BT) and increased uptake of ^{14}C-BT at 4°C. Moreover, tBOOH increased the efflux of ^{14}C-BT not mediated by breast cancer resistance protein. From these results, we conclude that tBOOH strongly inhibits SMCT1-mediated BT uptake, while having no effect on MCT1-mediated BT transport, and increases both uptake and efflux of BT through passive diffusion.

The conclusion that tBOOH inhibits SMCT1- but not MCT1-mediated BT uptake was based in the observation that tBOOH reduced Na^+-dependent but not Na^+-independent uptake of ^{14}C-BT by IEC-6 cells, together with the fact that tBOOH was devoid of effect upon ^{14}C-BT uptake in Caco-2 cells, which is mainly MCT1-mediated (Goncalves et al., 2009). Moreover, inhibition of uptake of only low concentrations of ^{14}C-BT also supports the conclusion that tBOOH inhibits SMCT1, because MCT1 has a low affinity/high transport capacity (K_m is about 2.6 mM BT) (Goncalves et al., 2009), and SMCT1 has a high affinity/low transport capacity (K_m is about 50 μM BT) (Thangaraju et al., 2008). SMCT1 depends on an intact Na^+ gradient which is maintained by the Na^+/K^+-ATPase (Coady et al., 2004). Interestingly, this may constitute a link between tBOOH and reduced SMCT1 activity, as ROS were found to reduce Na^+/K^+-ATPase membrane expression and activity (Thomas and Reed, 1990; Ogimoto et al., 2000).

We conclude that tBOOH increases BT uptake through passive diffusion because it increased uptake of ^{14}C-BT at 4°C, at which only noncarrier-medicated processes are operating, and also on the observation that uptake of high concentrations of ^{14}C-BT is increased by tBOOH at 37°C, a temperature at which both carrier and noncarrier-mediated mechanisms operate. As expected for a passive mechanism, this increase in diffusion of BT through the lipid bilayer in response to tBOOH is most evident for high concentrations of BT. Finally, the conclusion that tBOOH increases BT efflux not mediated by BCRP is based on the observation that tBOOH induced a Ko143-independent increase in ^{14}C-BT efflux. The stimulatory effect of tBOOH upon BT passive diffusion may well be related to the observed increase in lipid peroxidation caused by this agent. Moreover, it appears to be a specific effect, because tBOOH did not increase the efflux of another compound (5-methyltetrahydrofolate; results not shown).

Oxidative stress, and ROS in particular, can modulate transporter function by a number of mechanisms, including: 1) transcriptional regulation of gene expression; 2) changes in the gain in other signaling pathways that may in turn lead to changes in transporter activity or transporter gene expression; and 3) post-translational modifications of transporter structure (formation of intra- or inter-protein cross linked derivatives, peptide cleavage, nitrosylation, nitration, and oxidation of key amino acid residues with production of hydroxyl or carbonyl derivatives) (Matalon et al., 2003). From our qRT-PCR results, we conclude that tBOOH caused no changes in gene expression of MCT1 and SMCT1, the two main transporters involved in BT uptake by IEC-6 cells,

suggesting that oxidative stress affected transporter function at a post-transcriptional level. Moreover, the lack of effect of colchicine upon the inhibitory effect of tBOOH on ^{14}C-BT uptake points to the conclusion that tBOOH also did not affect the amount of protein transporters inserted in the cell membrane. On the contrary, our results suggest that tBOOH may be acting directly in protein structure, because tBOOH increased protein carbonylation. Protein carbonyl groups (aldehydes and ketones) are generated by direct oxidation of amino acid residues, particularly lysine, arginine, threonine, and proline (Dalle-Donne et al., 2003). Such modifications in proteins result in important changes in protein structure and possibly function. Of note, recently Simão et al. (Simao et al., 2011) demonstrated that ROS stimulate Cl^-/HCO_3^- exchanger activity through oxidation of thiol groups.

Because oxidative stress is known to activate several intracellular signaling pathways (Benhar et al., 2002; Liu et al., 2006; Poli et al., 2004; Wu, 2006), we decided to clarify the role of intracellular regulatory pathways in the effect of ^{14}C-BT uptake by tBOOH. These experiments showed that the inhibitory effect of tBOOH upon ^{14}C-BT uptake was partially reversed by genistein (but not by genistin) and by PD 98059, suggesting that inhibition of ^{14}C-BT uptake by tBOOH was dependent on PTK and MAPK ERK1/2 activation.

Inhibition of oxidative stress damage constitutes the first line of defense against carcinogenic insults and can be considered the most effective way for preventing cancer. Two different pharmacological approaches can be employed to counteract oxidative stress: selective inhibitors of various enzymatic sources of ROS, and antioxidant supplements (e.g., vitamins C, polyphenols).

ROS are produced through a variety of cellular oxidative metabolic processes, including NADPH oxidase, xanthine oxidase and arachidonic acid metabolism by cyclooxygenases and lipoxygenases (Kim et al., 2008). Because apocynin and allopurinol partially reversed the effect of tBOOH upon ^{14}C-BT uptake, inhibition of ^{14}C-BT uptake by tBOOH is dependent on generation of ROS by NADPH oxidase and xanthine oxidase; namely $O_2^{\bullet-}$ by NADPH oxidase, and $O_2^{\bullet-}$ and H_2O_2 by xanthine oxidase (Sato et al., 2011). Studies have demonstrated that pharmacological inhibition of NADPH oxidase may be more effective in modulating ROS production than scavenging of ROS by antioxidant supplements (Jaquet et al., 2009). So, use of NADPH and xanthine oxidase selective inhibitors can be useful to counteract the effect of oxidative stress upon ^{14}C-BT uptake.

Finally, we also investigated the ability of some antioxidants (N-acetyl-cysteine, vitamin C, epigallocatechin-3-gallate (EGCG), quercetin and resveratrol) to reduce/prevent the inhibition of ^{14}C-BT uptake caused by tBOOH. We verified that quercetin and resveratrol significantly decreased the inhibitory effect of tBOOH upon ^{14}C-BT uptake. Dietary polyphenols are considered to be antioxidants, due to their ability to directly scavenge ROS and to modulate the expression of antioxidant enzymes (Antosiewicz et al., 2008). Of these, quercetin and resveratrol were shown to have strong antioxidant activities (Kaindl et al., 2008; Pulido et al., 2000; Wolfe and Liu, 2007). Interestingly, the inhibitory effect of quercetin upon tBOOH-induced changes in ^{14}C-BT uptake correlated well with its inhibitory effect upon tBOOH-induced oxidative damage, as evaluated by quantification of lipid peroxidation.

5. Conclusion

Our results clearly show that oxidative stress decreases BT uptake by non-tumoral intestinal epithelial cells (Fig. 9). Given the important physiological role played by BT at the intestinal level, our results are indeed of major importance. Interaction of oxidative stress with BT transport is particularly important in the context of carcinogenesis and inflammatory bowel disease. Indeed, oxidative stress is associated with initiation and progression of colon carcinogenesis and inflammatory bowel disease (Acharya et al., 2010; Almenier et al., 2012; Seril et al., 2003), and is also involved in the link between chronic inflammation and cancer (Wiseman and Halliwell, 1996). BT has anticarcinogenic and antiinflammatory effect and reduces oxidative stress at the intestinal level (Hamer et al., 2008; Wong et al., 2006). So, given the protective role played by BT in the intestine, inhibition of BT intestinal epithelial uptake may contribute to the procarcinogenic and proinflammatory effect of oxidative stress at this level.

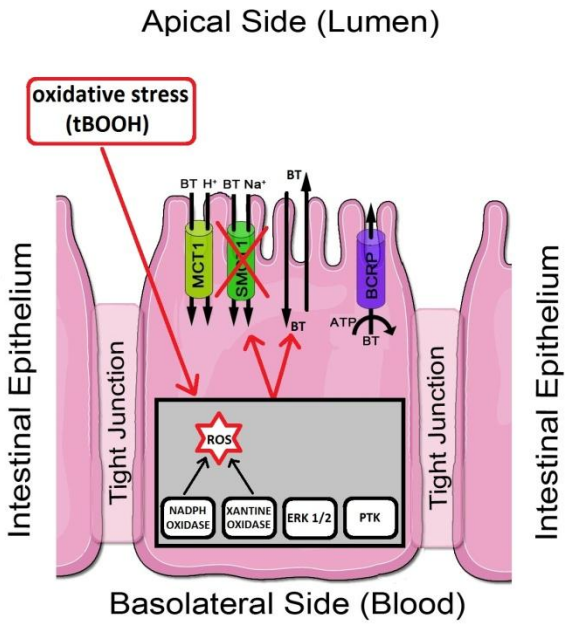

Figure 9. Effect of oxidative stress (tBOOH 3000 µM) on BT transport in intestinal epithelial cells. MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane. BCRP (gene name ABCG2) is an ATP dependent efflux transporter for BT at the apical membrane. Oxidative stress inhibits SMCT1-mediated BT uptake and stimulates both uptake and efflux of BT by passive diffusion. The inhibition of BT uptake is dependent on the generation of ROS by NADPH and xanthine oxidase, and on mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase 1/2 (ERK 1/2) and protein tyrosine kinase (PTK) activation.

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Declaration of Interest

No conflicts of interest are declared by the author(s).

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Lack of a Significant Effect of Cannabinoids upon the Uptake of 2-Deoxy-D-Glucose by Caco-2 Cells

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Key Words
Cannabinoids · 2-Deoxy-D-glucose · Caco-2 cells · Intestinal uptake

Abstract
The endogenous cannabinoid system plays a role in the regulation of energy homeostasis acting through central pathways, and its dysregulation may be implicated in the pathogenesis of obesity. Recent evidence is accumulating showing that the endogenous cannabinoid system is also present in peripheral tissues. The aim of this work was to investigate the effect of cannabinoids upon the intestinal absorption of glucose. For this, we investigated the effect of some cannabinoid receptor agonists and antagonists upon the apical uptake of ^{3}H-2-deoxy-D-glucose by the human intestinal epithelial Caco-2 cells. Uptake of a low concentration of ^{3}H-2-deoxy-D-glucose (1 μmol/l) was both cytochalasin B- and phloridzin-sensitive. The maximal inhibition obtained with each of these inhibitors was 50%, and their effect was not cumulative. On the other hand, uptake of a high concentration of ^{3}H-2-deoxy-D-glucose (20 mmol/l) was partially inhibited by cytochalasin B (±20%) and phloridzin had no effect. We verified that neither the cannabinoid receptor agonists [tetrahydrocannabinol (1–10 μmol/l), anandamide (0.1–10 μmol/l) and CP 55,940 (5 nmol/l to 1 μmol/l)], nor the specific CB_1 and CB_2 antagonists [AM251 (10–500 nmol/l) and AM630 (50 nmol/l to 1 μmol/l), respectively] had a significant effect upon ^{3}H-2-deoxy-D-glucose uptake by Caco-2 cells. This was true for both the uptake of a low (1 μmol/l) and of a high (20 mmol/l) concentration of ^{3}H-2-deoxy-D-glucose. From these results, we may hypothesize that cannabinoids do not interfere with the intestinal GLUT2-mediated apical uptake of glucose.

Introduction

Obesity has recently been declared a global epidemic by the World Health Organization. It constitutes a serious threat to public health because of its increased risk of associated health problems. The 1990 National Health and Nutrition Evaluation Survey, carried out by the US Government, demonstrated that one third of the adult population and over 20% of the children in the USA have medically significant obesity [1]. Obesity eventually leads to serious health consequences, including type 2 diabetes, coronary heart disease, hypertension, certain types of cancer, sleep apnea, bone joint diseases, nonalcoholic fatty liver disease, and psychological problems (reviews by Nisoli and Carruba [2] and Haslam and James [3]).

Marijuana, the female plant of *Cannabis sativa*, has been used in several forms (mostly smoked) as a recreational and medicinal agent for over 4,000 years [4, 5]. It contains several psychoactive, hallucinogenic chemicals with euphoric and sedative effects, termed cannabinoids [its main active ingredient is Δ^9-tetrahydrocannabinol

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(Δ^9-THC)]. Cannabinoids induce various neurobehavioral and neurochemical changes, including changes in memory, locomotion, and cognition [6]. Cannabinoids and their endogenous counterparts, the endocannabinoids, bind to G-protein-coupled cannabinoid (CB) type 1 and type 2 receptors. In animals, CB_1 is expressed in the brain, gastrointestinal organs and adipose tissue, whereas CB_2 is predominantly expressed on peripheral immune cells [7].

Recently, the endogenous cannabinoid system has been shown to be an important regulator of energy homeostasis: by activating central endocannabinoid CB_1 receptors, endocannabinoids [anandamide (N-arachidonoylethanolamine) and 2-arachidonoylglycerol] increase appetite, food consumption and promote weight gain [8–11]. In agreement with this, there is overactivation of the endocannabinoid system in genetic animal models of obesity [12, 13] and in obese humans and in humans with eating disorders [14, 15]. This phenomenon has been exploited in the treatment of cachexia using Δ^9-THC [6, 16]. Moreover, a synthetic Δ^9-THC analogue (dronabinol) is approved for use in the USA to stimulate appetite in AIDS wasting syndromes and chemotherapy [17, 18]. On the other hand, pharmacological CB_1 blockade with SR141716 (Rimonabant®) reduces food intake and body weight [11, 12, 19–21], and this drug has been tested successfully in phase III trials as an adjunctive obesity treatment [20–22].

Although a centrally mediated reduction in food intake forms the basis for the weight loss effect of CB_1 antagonists [20, 21], peripheral mechanisms appear to also contribute to this weight loss effect [11, 14]. For instance, CB_1 agonists increase hepatic lipogenesis [11, 23].

However, nothing is known concerning the effect of cannabinoids upon the intestinal absorption of glucose. Glucose is the main fuel that provides energy for normal activity in humans, its major source being the carbohydrate in food. Although not a nutritionally essential nutrient, glucose derived from food plays an important role in the regulation of normal plasma glucose levels after intestinal absorption. Plasma glucose levels, in turn, play a role in the control of food intake, and they also regulate insulin secretion by the pancreas. Peripheral insulin resistance (with its associated glucose intolerance) is closely linked to the development of the metabolic syndrome, a multifactorial condition leading to accelerated atherosclerosis and increased risk for diabetes, major cardiovascular events and a high mortality rate. So, interference with the intestinal absorption of glucose may also contribute to the effect of cannabinoids upon energy homeostasis. Interestingly enough, an important role of the endocannabinoid system in the gastrointestinal tract under physiological and pathophysiological conditions is well established (reviews by Massa et al. [24] and Sanger [25]).

Thus, the aim of this study was to investigate the effect of cannabinoids upon the intestinal absorption of glucose. For this, we tested the effect of several cannabinoids upon the apical uptake of ^{3}H-2-deoxy-D-glucose (^{3}H-DG) by Caco-2 cells. Caco-2 cells are an epithelial cell line derived from a human colon adenocarcinoma, which mimic the human intestinal absorptive epithelium [26].

Materials and Methods

Caco-2 Cell Culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage number 37 and 59. The cells were maintained in a humidified atmosphere of 5% CO_2/95% air and were grown in minimum essential medium (Sigma, St. Louis, Mo., USA) containing 5.55 mmol/l glucose and supplemented with 15% fetal calf serum, 25 mmol/l HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; ∅ 60 mm; Corning Costar, Corning, N.Y., USA). For uptake studies, Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar), and the experiments were performed 7–10 days after the initial seeding. The cell medium was free of fetal calf serum for 24 h before the experiments.

Determination of ^{3}H-DG Uptake by Caco-2 Cells

The uptake experiments were performed with Caco-2 cells incubated in glucose-free HEPES-buffered saline (containing 140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$ and 20 mmol/l HEPES, pH 7.4). Initially, the culture medium was aspirated and the cells were washed with 0.3 ml buffer at 37°C; then the cell monolayers were preincubated for 20 min in 0.3 ml buffer at 37°C. Uptake was initiated by the addition of 0.3 ml medium at 37°C containing 1 μmol/l or 20 mmol/l ^{3}H-DG. At the end of the incubation period, incubation was stopped by placing the cells on ice and rinsing the cells with 0.3 ml ice-cold buffer. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mmol/l Tris-HCl, pH 7.4), and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Effect of Drugs

Drugs to be tested were present during both the preincubation and incubation periods.

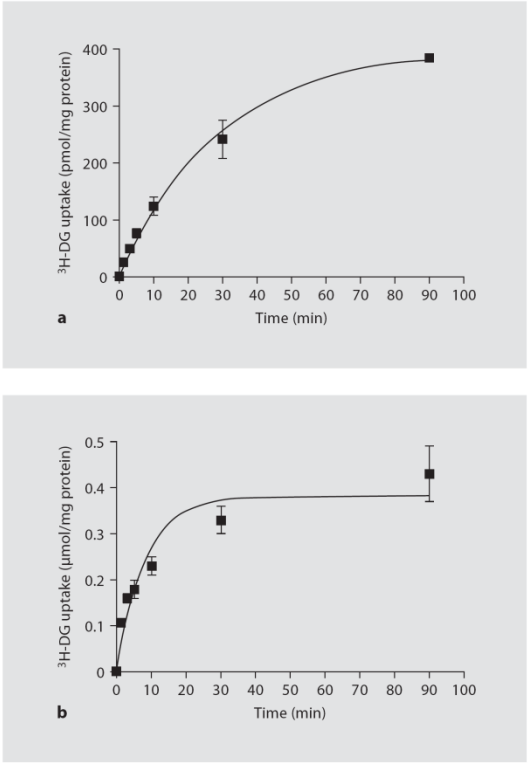


Fig. 1. Time course of ^{3}H-DG apical uptake by Caco-2 cells. Caco-2 cells were incubated at 37°C with 1 µmol/l (**a**) or 20 mmol/l (**b**) ^{3}H-DG. Shown are arithmetic means ± SEM (n = 4).

Protein Determination

The protein content of cell monolayers was determined as described by Bradford [27], using human serum albumin as standard.

Calculation and Statistics

For the analysis of the time course of ^{3}H-DG uptake, the parameters of equation 1 were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method [28].

$$A(t) = k_{in}/k_{out} (1 - e^{-k_{out} \cdot t}) \quad (1)$$

A(t) represents the accumulation of ^{3}H-DG at time t, k_{in} and k_{out} the rate constants for inward and outward transport, respectively, and t the incubation time. A_{max} corresponds to the accumulation [A(t)] at steady state ($t \rightarrow \infty$). k_{in} is given in pmol mg^{-1} protein min^{-1} and k_{out} in min^{-1}. In order to obtain clearance values, k_{in} was converted to µl mg^{-1} protein min^{-1}.

For the analysis of the saturation curve of ^{3}H-DG uptake, the parameters of the Michaelis-Menten equation were fitted to the experimental data by a nonlinear regression analysis, using a computer-assisted method [28].

Arithmetic means are given with SEM. Statistical significance of the difference between various groups was evaluated by Student's t test. Differences were considered to be significant when $p < 0.05$.

Materials

^{3}H-DG (deoxy-D-glucose, 2-[1,2-^{3}H]; specific activity 50 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK); (–)-Δ^9-THC (tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo [b,d]pyran-1-ol; Cerilliant Corporation, Round Rock, Tex., USA), AM251 [(N-[piperidin-1-yl]-5-[iodophenyl]-1-82,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], AM630 [(6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl)(4-methoxyphenyl)methanone], anandamide [N-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide], CP 55,940 [(–)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; Tocris Cookson Inc., Ellisville, Mo., USA], cytochalasin B (from *Drechslera dematioidea*), 2-deoxy-D-glucose, phloridzin dihydrate, and trypsin-EDTA solution (Sigma, St. Louis, Mo., USA) were used.

Results

Time Course of ^{3}H-DG Uptake

In a first series of experiments, we determined the time course of ^{3}H-DG apical uptake by Caco-2 cells. For this, cells were incubated with two distinct concentrations of ^{3}H-DG (1 µmol/l or 20 mmol/l) for various periods of time. As shown in figure 1, Caco-2 cells accumulated 1 µmol/l and 20 mmol/l ^{3}H-DG in a time-dependent way.

Analysis of the time course of ^{3}H-DG accumulation showed that, for 1 µmol/l ^{3}H-DG, k_{in} was 13.9 ± 1.3 µl mg^{-1} protein min^{-1}, k_{out} was 0.035 ± 0.005 min^{-1} and A_{max} was 396.2 ± 21.1 pmol mg^{-1} protein. For 20 mmol/l ^{3}H-DG, k_{in} was 2.4 ± 0.2 µl mg^{-1} protein min^{-1}, k_{out} was 0.124 ± 0.025 min^{-1} and A_{max} was 381.4 ± 26.6 nmol mg^{-1} protein.

Both at the low (1 µmol/l) and high (20 mmol/l) concentration of ^{3}H-DG, uptake was linear for up to 10 min of incubation (fig. 1). So, in all subsequent experiments, cells were incubated with ^{3}H-DG for 6 min, in order to measure initial rates of uptake.

Kinetics of ^{3}H-DG Uptake

Next, the kinetics of ^{3}H-DG uptake by Caco-2 cells was examined. For this, cells were incubated for 6 min

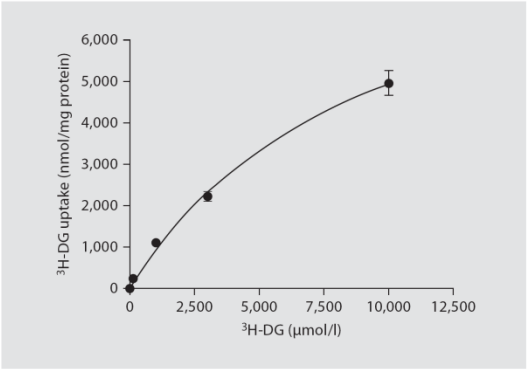


Fig. 2. Uptake of ^{3}H-DG as a function of substrate concentration. Caco-2 cells were incubated at 37°C, for 6 min, in the presence of increasing concentrations of ^{3}H-DG (1 μmol/l to 10 mmol/l). Shown are arithmetic means ± SEM (n = 5–6).

with increasing (1 μmol/l to 10 mmol/l) concentrations of ^{3}H-DG (fig. 2). Analysis of the saturation curve showed that uptake followed a single Michaelis-Menten kinetics, with a K_m of 9.08 ± 1.61 mmol/l and a V_{max} of 9,405 ± 935 nmol mg^{-1} protein 6 min^{-1} (n = 5–6).

Effect of Glucose Transport Inhibitors on ^{3}H-DG Uptake

The specificity of ^{3}H-DG uptake was next investigated. For this, the effects of cytochalasin B (an inhibitor of facilitated glucose transporters, GLUTs) and phloridzin (a specific inhibitor of sodium-linked glucose transporters, SGLTs) were evaluated (fig. 3).

Interestingly, uptake of a low concentration of ^{3}H-DG (1 μmol/l) was inhibited by both phloridzin and cytochalasin B, both maximally inhibiting ^{3}H-DG uptake by 50%. However, a combination of these two compounds did not result in a greater inhibition of ^{3}H-DG uptake (fig. 3a).

On the other hand, uptake of a high concentration of ^{3}H-DG (20 mmol/l) was inhibited by cytochalasin B, but not by phloridzin. Moreover, the maximal inhibition obtained with cytochalasin B was rather small (about 15%) (fig. 3b).

Effect of Cannabinoids on ^{3}H-DG Uptake

In the next series of experiments, the effect of some cannabinoid agonists was tested.

Uptake of a low concentration of ^{3}H-DG (1 μmol/l) was unaffected by any of the cannabinoid receptor ago-

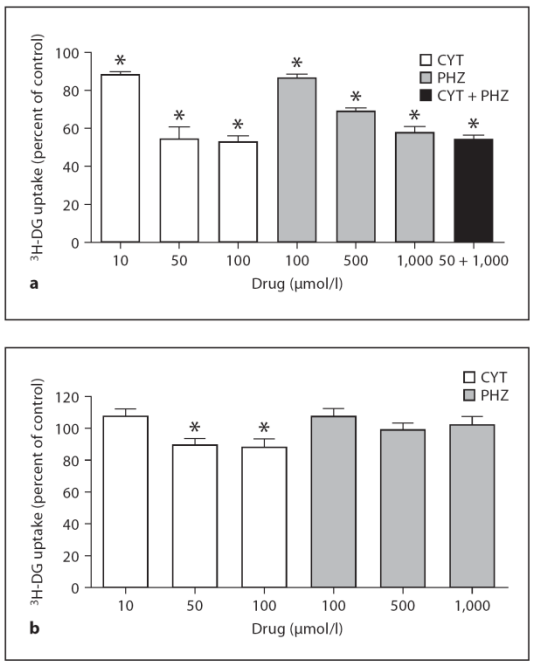
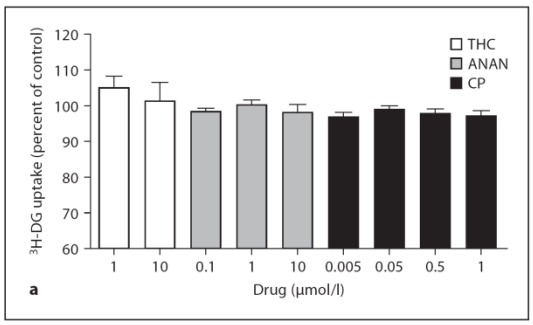
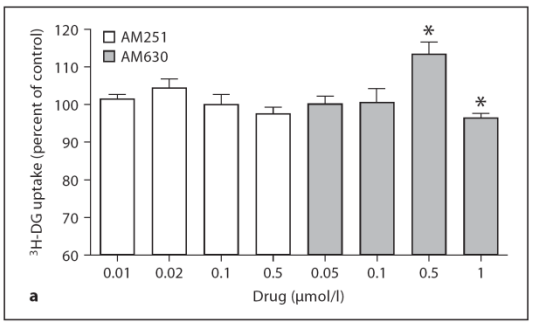


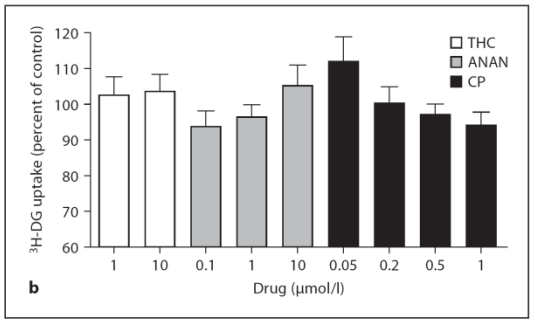
Fig. 3. Effect of cytochalasin B (CYT) and phloridzin (PHZ) on the uptake of ^{3}H-DG 1 μmol/l (**a**) or 20 mmol/l (**b**) by Caco-2 cells. Caco-2 cells were incubated at 37°C with ^{3}H-DG, for 6 min, in the absence or presence of increasing concentrations of these drugs. CYT + PHZ: CYT 50 μmol/l + PHZ 1 mmol/l. Shown are arithmetic means ± SEM (n = 5–12). * $p < 0.05$: significantly different from the respective control.

nists tested, namely THC (1 and 10 μmol/l), anandamide (0.1–10 μmol/l) and CP 55,940 (0.005–1 μmol/l) (fig. 4a). Similarly, uptake of a saturating concentration of ^{3}H-DG (20 mmol/l) was also unaffected by any of the agonists tested (THC, anandamide and CP 55,940) (fig. 4b).

Next, the effect of two selective inhibitors of the cannabinoid receptors CB_1 and CB_2 (AM251 and AM630, respectively) was evaluated. AM630 (500 nmol/l) caused an increase (of 13%) in the uptake of a low concentration of ^{3}H-DG (1 μmol/l), but a higher concentration of AM630 (1 μmol/l) decreased ^{3}H-DG uptake by about 4%. AM251 was devoid of effect (fig. 5a). In relation to uptake of a high (20 mmol/l) concentration of ^{3}H-DG, it was slightly inhibited by both AM251 (20 nmol/l) and AM630 (0.5 and 1 μmol/l) (fig. 5b).







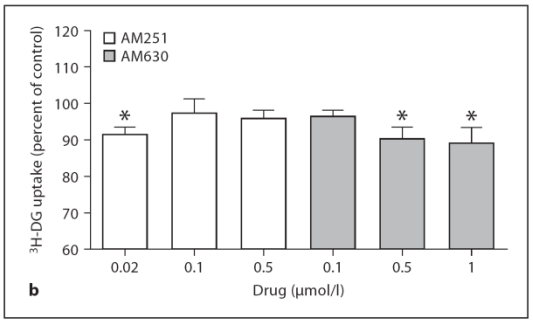


Fig. 4. Effect of the cannabinoid agonists Δ^9-THC (THC; n = 8–11), anandamide (ANAN; n = 8–12) and CP 55,940 (CP; n = 3–17) on the uptake of ^{3}H-DG 1 μmol/l (**a**) or 20 mmol/l (**b**) by Caco-2 cells. Caco-2 cells were incubated at 37°C with ^{3}H-DG, for 6 min, in the absence or presence of increasing concentrations of these drugs. Shown are arithmetic means ± SEM. * $p < 0.05$: significantly different from the respective control.

Fig. 5. Effect of the cannabinoid antagonists AM251 (n = 9–18) and AM630 (n = 6–9) on the uptake of ^{3}H-DG 1 μmol/l (**a**) or 20 mmol/l (**b**) by Caco-2 cells. Caco-2 cells were incubated at 37°C with ^{3}H-DG, for 6 min, in the absence or presence of increasing concentrations of these drugs. Shown are arithmetic means ± SEM. * $p < 0.05$: significantly different from the respective control.

Finally, the influence of the two cannabinoid receptor antagonists, AM251 (20 and 100 nmol/l) and AM630 (500 nmol/l), on the effect of THC (1 μmol/l) or CP 55,940 (200 nmol/l) upon the uptake of a saturating concentration of ^{3}H-DG (20 mmol/l) was tested. In these experiments, the antagonists were present for 20 min, after which a normal preincubation and incubation period followed. None of the antagonists was able to significantly modify the effect of CP 55,940 (500 nmol/l) or THC (1 μmol/l) (results not shown).

Discussion

The aim of this study was to investigate the effect of cannabinoids upon the intestinal absorption of glucose. For this purpose, we tested the effect of some cannabinoid receptor agonists and antagonists upon the apical uptake of ^{3}H-DG by Caco-2 cells.

Glucose is the main source of calories for normal activity in humans, and it is mainly derived from complex carbohydrates present in food. These complex carbohydrates are digested to monosaccharides, mostly glucose, prior to absorption in the small intestine. According to the 'classical model of sugar absorption', glucose is actively taken up into the enterocytes from the intestinal lumen by a high-affinity, Na^+-dependent and phloridzin-sensitive glucose cotransporter (SGLT1) located in the brush border and is then passively released from the enterocytes into the circulation via an Na^+-independent glucose transporter (GLUT2) present in the basolateral membrane (reviews by Kellett and Brot-Laroche [29], Drozdowski and Thompson [30] and Wright et al. [31]). The Na^+-cou-

pled glucose cotransporter SGLT1 belongs to the SLCA5 family of transporters, and actively transports glucose and galactose with similar and high affinities (around 0.1–0.6 mmol/l) [31–33]. On the other hand, GLUT2 is a low-affinity, high-capacity facilitative glucose, fructose and galactose transporter belonging to the SLC2 gene family [30, 34, 35]. However, this model has recently been challenged by a number of investigators. At the brush border membrane, several lines of evidence demonstrate that there are two types of glucose transporter: one is SGLT1, and the other is a low-affinity transporter which may, or may not, be Na^+-dependent and phloridzin-sensitive (reviews by Drozdowski and Thompson [30], Wright et al. [31] and Kellett [36]). For this second transport activity, some authors favor the existence of a facilitated (GLUT2) transporter, i.e., an Na^+-independent and phloretin-sensitive carrier with a glucose K_m in excess of 25 mmol/l (reviews by Kellett and Brot-Laroche [29], Drozdowski and Thompson [30], Wright et al. [31] and Kellett [36]). Interestingly enough, the functional evidence for the presence of apical GLUT2 in the intestine has recently been corroborated by the detection of GLUT2 in the brush border membrane by immunocytochemistry [37–39].

Apical GLUT2 is activated by meals, and the apical GLUT2 component of absorption is several times greater than the active component at high glucose concentrations. Moreover, apical GLUT2 is primed by a long-term diet containing high-glycemic-index sugars, and a low-glycemic-index diet decreases apical GLUT2 (reviews by Kellett and Brot-Laroche [29] and Kellett [36]). So, we decided to investigate the effect of cannabinoids upon the apical GLUT2-mediated transport using, for that purpose, DG as a model substrate. DG is a D-glucose analogue that is transported efficiently by facilitated glucose transporters such as GLUT1 and GLUT2, but is poorly transported by SGLT1 [31].

We verified that uptake of a low concentration of ^{3}H-DG (1 μmol/l) was partially inhibited by both cytochalasin B and phloridzin. The maximal inhibition obtained with each of these inhibitors was 50%, and their effect was not cumulative. On the other hand, uptake of a high concentration of ^{3}H-DG (20 mmol/l) was partially inhibited by cytochalasin B (±20%), phloridzin having no effect. Considering that cytochalasin B is a specific inhibitor of GLUT2 (see review by Drozdowski and Thompson [30]), these results point to the involvement of GLUT2 in the apical uptake of both the low and high concentration of ^{3}H-DG. Moreover, phloridzin is a specific inhibitor of SGLT1-mediated transport, but we do not believe that SGLT1 is involved in the uptake of a low concentration of ^{3}H-DG. First, DG is known to be a very poor substrate of SGLT1 [36]. Second, the inhibitory effects of phloridzin and cytochalasin B on ^{3}H-DG uptake were not cumulative, suggesting that both compounds are acting on the same transporter. However, we also do not believe that cytochalasin B is inhibiting SGLT1, or phloridzin is inhibiting GLUT2, because we could not find any reference in the literature to this possibility. Rather, these compounds are widely used as GLUT2- and SGLT1-specific inhibitors, respectively. So, at the present moment, we cannot explain the simultaneous inhibition of ^{3}H-DG (1 μmol/l) uptake by both cytochalasin B and phloridzin. Interestingly enough, Bissonnette et al. [40] observed that the apical uptake of DG in Caco-2 cells involves 2 distinct mechanisms: (1) an Na^+-independent and phloretin-sensitive system transporting DG (but not 3-O-methyl-D-glucose), and (2) an Na^+-dependent, phloridzin- and phloretin-sensitive system transporting both DG and 3-O-methyl-D-glucose. The involvement of SGLT1 in the uptake of DG was ruled out. Moreover, they noted that the second mechanism was constitutively expressed in all Caco-2 cells. So, although these authors did not advance the nature of these two transport mechanisms, they also verified that the uptake of DG was phloridzin-sensitive.

We tested the effect of some cannabinoid receptor agonists, namely THC, anandamide and CP 55,940, upon the uptake of ^{3}H-DG. Anandamide is an endogenous cannabinoid and vanilloid receptor agonist (K_i = 89 and 371 nmol/l at the CB_1 and CB_2 receptors, respectively) [41, 42]. Both Δ^9-THC (the main psychoactive component of marijuana) and CP 55,940 (a synthetic cannabinoid analogue) display a roughly equal affinity for both cannabinoid receptors, but CP 55,940 is considerably more potent than Δ^9-THC (K_i = 0.6–5.0 and 0.7–2.6 nmol/l at the CB_1 and CB_2 receptors, respectively) [43–45]. We also tested the effect of two selective CB receptor antagonists: AM251 and AM630. AM251 is a potent and selective CB_1 receptor antagonist/inverse agonist. It displays a K_i value of 7.49 nmol/l at CB_1 receptors and is 306-fold selective over CB_2 receptors [43, 46, 47]. On the other hand, AM630 is a CB_2 antagonist/inverse agonist (K_i = 31.2 nmol/l), and 165-fold selective over CB_1 receptors [48, 49].

We verified that neither the cannabinoid receptor agonists (THC, anandamide and CP 55,940) nor the specific CB_1 and CB_2 receptor antagonists (AM251 and AM630, respectively) had a significant effect upon ^{3}H-DG (1 μmol/l and 20 mmol/l) uptake by Caco-2 cells.

Interestingly enough, cannabinoids have recently been shown to inhibit the Na^+-dependent, high-affinity excitatory amino acid transporter [50], and the plasmalemmal

serotonin [51] and dopamine [52] transporters. Cannabinoids also interfere with P-glycoprotein [53], with an equilibrative nucleoside transporter [54], and with the placental uptake of the vitamin folate [55].

In relation to the effect of cannabinoids upon glucose uptake, it has recently been described that a 7-day treatment with the selective CB_1 receptor antagonist SR141716 increased (by 68%) glucose uptake in the isolated soleus muscle of the *Lep*^{ob}/*Lep*^{ob} mice [56]. In another study, a 24-hour treatment with anandamide was found to increase (by 2-fold) the insulin-stimulated glucose (2-deoxy-D-glucose) uptake, but not the basal uptake, in differentiated mouse adipocytes [57]. Finally, a 30-min exposure to the cannabinoid CB_1 receptor agonist Win 55,212 increased (by 50%) basal glucose (2-deoxy-D-glucose) uptake and the translocation of GLUT4 in human fat cells, but had no effect upon the insulin-stimulated glucose uptake [58]. So, the effect of cannabinoids upon glucose uptake by muscle and fat cells, which is probably mainly GLUT4 mediated, is rather ambiguous.

Recent reports have established that, in rats, cannabinoid CB_1 and CB_2 receptors modulate glucose homeostasis after a glucose load [59, 60]. Moreover, Rimonabant has been shown to lower plasma insulin and correct insulin resistance [61]. From our results, we think that a contribution of a reduced intestinal absorption of glucose to the effect of cannabinoids upon glucose homeostasis is not to be expected.

In conclusion, neither cannabinoid agonists nor antagonists had a significant effect upon the uptake of ^{3}H-DG by Caco-2 cells. This was true for both the uptake of a low and of a high concentration of ^{3}H-DG. So, we may hypothesize that cannabinoids do not interfere with the intestinal GLUT2-mediated apical uptake of glucose, both when a low concentration of ^{3}H-DG is present in the intestinal lumen (e.g. during the overnight period) as well as when a high concentration of ^{3}H-DG is present in the intestinal lumen (e.g. in the absorptive period).

Acknowledgements

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X - The effect of clotrimazole on energy substrate uptake and carcinogenesis in intestinal epithelial cells

Preclinical report 1

The effect of clotrimazole on energy substrate uptake and carcinogenesis in intestinal epithelial cells

Pedro Gonçalves, Inês Gregório, João R. Araújo and Fátima Martel

Clotrimazole has anticarcinogenic activity in several cell types. Our aims were to investigate the anticarcinogenic effect of clotrimazole in a tumoral intestinal epithelial (Caco-2) cell line, to compare it with the effect in a nontumoral intestinal epithelial cell line (IEC-6 cells), and to investigate inhibition of energy substrate uptake as a mechanism contributing to it. The effect of clotrimazole on cell proliferation, viability and differentiation, ^{3}H-deoxyglucose (^{3}H-DG), ^{3}H-O-methyl-glucose (^{3}H-OMG), and ^{14}C-butyrate uptake, as well as mRNA expression levels of glucose transporters was assessed. In Caco-2 cells, clotrimazole decreased cellular viability and proliferation and increased cell differentiation. The effect on cell proliferation and viability was potentiated by rhodamine123. Clotrimazole also decreased cellular viability and proliferation in IEC-6 cells, but increased the cellular DNA synthesis rate and had no effect on cell differentiation. Exposure of Caco-2 cells to clotrimazole (10 μmol/l) for 1 and 7 days increased (by 20–30%) the uptake of ^{3}H-DG and ^{3}H-OMG, respectively, but had no effect on ^{14}C-butyrate uptake. The effect on ^{3}H-DG and ^{3}H-OMG transport was maximal at 10 μmol/l, and the pharmacological characteristics of transport were not changed. However, clotrimazole changed the mRNA expression levels of the facilitative glucose transporter 2 and the Na^{+}-dependent glucose cotransporter. Clotrimazole exhibits comparable cytotoxic effects in tumoral and nontumoral intestinal epithelial cell lines. In Caco-2 cells, the cytotoxic effect of clotrimazole was strongly potentiated by the inhibition of oxidative phosphorylation. Moreover, stimulation of glucose uptake might be a compensation mechanism in response to the glycolysis inhibition caused by clotrimazole. *Anti-Cancer Drugs* 00:000–000 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: anticarcinogenic effect, butyrate, Caco-2 cells, cell membrane transport, clotrimazole, glucose, IEC-6 cells

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Introduction

Colorectal cancer is one of the most common solid tumors worldwide, being the second leading cause of cancer death among men and women combined in occidental countries [1].

Clotrimazole is an antifungal imidazole derivative in clinical use for more than 20 years. It was also found to act as a calmodulin antagonist and to have anticancer properties against different types of cancer (e.g. [2–10]). Its antineoplastic properties are associated with the ability to decrease glucose consumption and energy metabolism in tumor cells by inhibiting glycolysis and ATP production. More specifically, clotrimazole causes detachment of the glycolytic enzymes, hexokinase (from the mitochondrial membrane) and phosphofructokinase-1 and aldolase (from the cytoskeleton), originating a decrease in glycolysis, with a corresponding reduction in the cellular ATP content and cancer cell viability (e.g. [2–9]). Moreover, direct inhibition of phosphofructokinase-1 was also reported [10].

At the intestinal level, the anticancer effect of clotrimazole was described previously only in a murine colon adenocarcinoma cell line (CT-26 cells) [5,6] and in a serum-independent HT29-S-B6 human colon adenocarcinoma cell clone [11]. Therefore, we found it important to investigate its anticarcinogenic effect in the widely used human Caco-2 colon adenocarcinoma cell line [12] and to characterize this effect.

For such a characterization, we first decided to compare it with the effect in a nontumoral epithelial intestinal cell line, the IEC-6 cells [13]. Comparison between the effect of clotrimazole in a carcinogenic and a noncarcinogenic cell line seemed interesting in the context of a possible distinct effect of clotrimazole in these cells.

Glycolysis is the primary energy source for cancer cells, exceeding the capacity of mitochondrial oxidative energetic metabolism [14–16]. However, in some tumor cells, the sole application of glycolytic drugs does not decrease tumor progression significantly, but when these are combined with an inhibitor of mitochondrial oxidative phosphorylation (e.g. rhodamine123), the proliferation rate is decreased drastically [17–19]. Therefore, we also decided to evaluate the anticarcinogenic effect of clotrimazole in conjunction with rhodamine123 in Caco-2 cells.

Finally, we decided to analyze the effect of clotrimazole on glucose and butyrate uptake by Caco-2 cells as a possible mechanism contributing to its anticarcinogenic effect. Glucose transport was studied using two

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analogues: 2-deoxyglucose (DG) and 3-O-methylglucose (OMG). DG is a glucose analogue efficiently transported by facilitative glucose transporters such as GLUT1 and GLUT2, but poorly transported by the sodium-dependent glucose transporter SGLT1 [20]. In contrast, OMG is a substrate for both SGLT1 and GLUT2 [20].

Butyrate, a product of intestinal flora fermentation of dietary fiber, is an important metabolic substrate in normal colonic epithelial cells. However, butyrate becomes less essential for growth of neoplastic cells, which are highly glycolytic, producing excessive lactic acid. Indeed, colonic carcinomas show a reduction in butyrate uptake, and glycolysis becomes the primary energy source, exceeding the capacity of mitochondrial oxidative energetic metabolism [14–16]. Interestingly enough, butyratealso has a protective role in the prevention and progression of colorectal carcinogenesis [21], and the mechanisms involved in its cellular uptake have been proposed to function as tumor suppressors [21,22].

Materials and methods

Caco-2 and IEC-6 cell culture

The Caco-2 and IEC-6 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passage numbers 53–71 (Caco-2 cells) and 30–41 (IEC-6 cells). The cells were maintained in a humidified atmosphere of 5% CO_2–95% air. Caco-2 cells were cultured in minimum essential medium containing 5.55 mmol/l of glucose and supplemented with 15% fetal calf serum, 25 mmol/l of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B. IEC-6 cells were cultured in Dulbecco's Modified Eagle's Medium:Roswell Park Memorial Institute Medium 1640 medium (1 : 1), supplemented with 10% fetal bovine serum, 0.1 U/ml of insulin, 5.96 g of HEPES, 2.2 g of $NaHCO_3$, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B. The culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1 : 3, and subcultured in plastic culture dishes (21 cm^2; 60-mm diameter; Corning Costar, Corning, New York, USA). For use in experiments, cells were seeded on 24-well plastic cell culture clusters (2 cm^2; 16-mm diameter; Techno Plastic Products), and experiments were performed 7–8 days after the initial seeding (90–100% confluence).

Treatment of the cells with clotrimazole *or rhodamine123*

The effect of clotrimazole was tested by cultivating cells in culture medium containing clotrimazole (or the respective solvent) for 1, 2, 3, or 7 days. The effect of rhodamine123 was tested by cultivating cells in culture medium containing rhodamine123 or the respective solvent for 7 days. The medium was renewed daily, and the end of the treatment period was always day 8 of cell culture.

Quantification of cellular viability (lactate dehydrogenase assay)

At the end of the treatment period (7 days), leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium was measured spectrophotometrically [23]. In brief, extracellular LDH activity was quantified by measuring, in the culture medium, the oxidation of NADH at a wavelength of 340 nm during the reduction of pyruvate to lactate. Optical density values were determined for 2 min, and the rate of NADH oxidation was then calculated. To determine the total LDH activity, cells from control cultures were solubilized with 0.5 ml 0.1% (v/v) Triton X-100 (in mmol/l Tris–HCl, pH 7.4), and placed for 30 min at 37°C. The amount of LDH present in the extracellular medium, which correlates with cell death, was then calculated as a percentage of the total LDH activity.

Determination of cellular proliferation

Cellular proliferation was quantified by two different methods: quantification of the whole-cell protein [sulforhodamine B (SRB) assay] and quantification of the cellular DNA synthesis rate (^{3}H-thymidine incorporation assay).

Quantification of the whole-cell protein (sulforhodamine B assay)

After the treatment period (7 days), 62.5 μl of ice-cold 50% (w/v) trichloroacetic acid (TCA) were added to the culture medium (500 μl) in each well to fix cells (1 h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed, and the cultures were rinsed four times with 1% (v/v) acetic acid to remove the residual dye. Plates were again air-dried, and the bound dye was then solubilized with 375 μl of 10 mmol/l Tris: NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted in order to obtain absorbance values lower than 0.7.

Quantification of ^{3}H-thymidine incorporation

Quantification of the cellular DNA synthesis rate was obtained by measuring the incorporation of ^{3}H-thymidine into cellular DNA. After the treatment period (7 days), 500 μl of culture medium at 37°C containing ^{3}H-thymidine (0.25 μCi/ml) (and clotrimazole or the respective solvent) was added for 5 h. After this period, the medium was removed and the cells were fixed by incubation with 300 μl 10% TCA (1 h at 4°C). Then, the cells were washed twice with 10% TCA to remove the unbound radioactivity,

plates were air-dried for 30 min, and finally the cells were lysed with 1 mol/l NaOH (280 µl/well). A 250 µl aliquot of the lysate was neutralized with HCl before the addition of the scintillation fluid. The radioactivity of the samples was quantified by liquid scintillation counting. The cellular DNA synthesis rate was expressed as incorporation of ^{3}H-thymidine/well.

Determination of cellular differentiation (alkaline phosphatase activity assay)

After the treatment period (7 days), cell differentiation was determined by quantification of the alkaline phosphatase activity, as described previously [24]. In brief, cells were lysed and solubilized with 250 µl of Triton X-100 at 4°C. The enzymatic reaction was started by adding *p*-nitrophenylphosphate as a substrate. The reaction mixture contained, in a final volume of 500 µl: 80 mmol/l of Tris–HCl (pH 10.2), 0.4 mmol/l of $MgCl_2$, 0.376 mg of *p*-nitrophenylphosphate, and the cell sample (200 µl). Incubation took place at 37°C for 60 min, was stopped by the addition of 2 ml:20 mmol/l of NaOH (ice cold), and then the reaction mixture was placed on ice for 4 min. At the end of this period, the absorbance of *p*-nitrophenol (405 nm), produced by the hydrolysis of *p*-nitrophenylphosphate, was measured spectrophotometrically (Multiskan Ascent, Thermo Scientific). The enzyme activity, calculated as nmol *p*-nitrophenol/min/mg protein, was expressed as a percentage of the control.

Uptake of energy substrates in Caco-2 cells

Uptake experiments were performed with Caco-2 cells incubated in glucose-free Krebs buffer containing the following (in mmol/l): 125 mmol/l of NaCl, 4.8 mmol/l of KCl, 1.2 mmol/l of $MgSO_4$, 1.2 mmol/l of $CaCl_2$, 25 mmol/l of $NaHCO_3$, 1.6 mmol/l of KH_2PO_4, 0.4 mmol/l of K_2HPO_4, and 20 mmol/l of HEPES, pH 7.4 [^{3}H-deoxyglucose (^{3}H-DG) and ^{3}H-O-methyl-glucose (^{3}H-OMG) experiments], or 20 mmol/l of 2-(*N*-morpholino)ethanesulfonic acid hydrate, pH 6.5 (^{14}C-BT experiments) [25,26].

In all the experiments, the culture medium was aspirated and the cells were washed twice with 0.3 ml of buffer at 37°C. Then, the cell monolayers were incubated with 0.3 ml medium at 37°C, containing ^{14}C-BT (10 µmol/l), ^{3}H-DG (1 µmol/l), or ^{3}H-OMG (10 µmol/l). Incubation was stopped after 3 min (^{14}C-BT and ^{3}H-OMG) or 6 min (^{3}H-DG) by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.5 ml ice-cold buffer. The cells were then solubilized with 0.3 ml of 0.1% (v/v) Triton X-100 (in 5 mmol/l of Tris–HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting. For the pharmacological characterization of ^{3}H-DG and ^{3}H-OMG uptake with glucose transport inhibitors, the cell monolayers were preincubated for 20 min in 0.3 ml of buffer at 37°C before the incubation with either ^{3}H-DG or ^{3}H-OMG. Glucose transport inhibitors to be tested were present during both the preincubation and the incubation periods.

Protein determination

The protein content of cell monolayers was determined as described [27], using human serum albumin as the standard.

Real-time quantitative reverse transcription polymerase chain reaction

Caco-2 cells were treated for 1 or 7 days with clotrimazole (10 µmol/l or the respective solvent). Then, the total RNA was extracted from cells using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Basel, Germany).

Before cDNA synthesis, the total RNA was treated with DNase I (Invitrogen Corporation, California, USA) according to the manufacturer's instructions, and 10 µg of the resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 40 µl of the final reaction volume, according to the manufacturer's instructions. The resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade the unreacted RNA. For the quantitative real-time polymerase chain rection, 2 µl of the 40-µl reverse transcription reaction mixture was used. For the calibration curve, Caco-2 standard cDNA was diluted in five different concentrations.

Real-time polymerase chain rection was carried out using a LightCycler (Roche, Nutley, New Jersey, USA). Reactions of 20 µl were set up in microcapillary tubes using 0.5 µmol/l of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). The cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 15 s, and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10-s segment] repeated 40 times, a melting curve program [(AT + 10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement], and a cooling step to 40°C (30 s). The primer pairs used for amplification and the ATs were as follows: 5′-ATG GAG AAG GCT GGG GCT CAT-3′ (forward) and 5′-GAC GAA CAT GGG GGC ATC AG-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (AT, 65°C); 5′-TGG CAA TCA CTG CCC TTT AT-3′ (forward) and 5′-TGC AAG GTG TCC GTG TAA AT-3′ (reverse) for human sodium-dependent glucose transporter type 1 (SGLT1; AT, 60°C); and 5′-CAG GAC TAT ATT GTG GGC TAA-3′ (forward) and 5′-CTG ATG AAA AGT GCC AAG T-3′ (reverse) for human facilitative glucose transporter type 2 (GLUT2; AT 65°C). Quantification of mRNA levels was performed by fluorescence measurement using the LightCycler 4.05 analysis software (Roche, Mannheim, Germany).

Calculation and statistics

Three independent experiments were performed, at least in triplicate, except in experiments with glucose transport inhibitors, where one experiment was performed in triplicate. Arithmetic means are given with SEM or SD. Statistical significance of the difference between two groups was evaluated by one-tailed Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance test, followed by the Bonferroni test. Differences were considered to be significant when a *P* value of less than 0.05.

Materials

^{14}C-BT [(1-^{14}C)-*n*-butyric acid, sodium salt; specific activity 30–60 mCi/mmol), ^{3}H-DG {2-[1,2-^{3}H(N)]-DG; specific activity 50 Ci/mmol}, ^{3}H-OMG [(metil-^{3}H)-OMG; specific activity 80 Ci/mmol; Biotrend Chemikalien GmbH, Koln, Germany], ^{3}H-thymidine [methyl-(^{3}H)-thymidine; specific activity 79 Ci/mmol; GE Healthcare GmbH, Freiburg, Germany], clotrimazole, cytochalasin B (from *Diechslera dematioidea*), dimethylsulfoxide, Dulbecco's Modified Eagle's Medium: Roswell Park Memorial Institute Medium 1640 medium (1 : 1), ethanol, HEPES, MES, penicillin/streptomycin/amphotericin B solution, phloridzin dehydrate, rhodamine123 hydrate, sulforhodamine B, trichloroacetic acid sodium salt, triton X-100, trypsin-EDTA solution (Sigma, St. Louis, Missouri, USA), and fetal calf serum (Gibco, California, USA).

The drugs to be tested were dissolved in dimethylsulfoxide or ethanol; the final concentration of these solvents in the culture medium was 1%. Controls for these drugs were run in the presence of the respective solvent.

Results

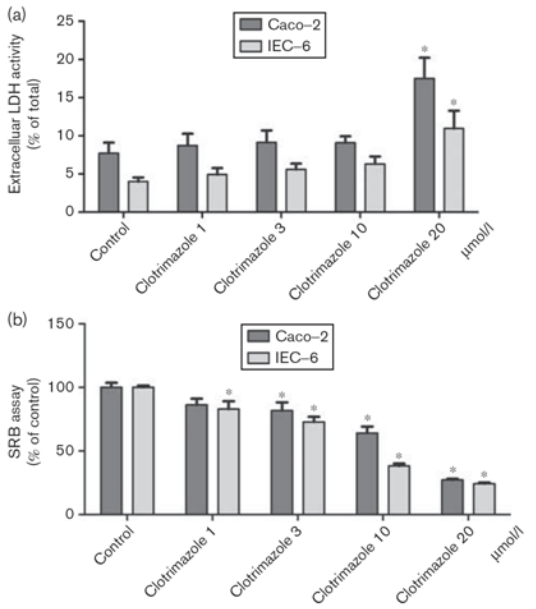
Effect of clotrimazole *on the cellular viability, proliferation, and differentiation of Caco-2 and IEC-6 cells*

In the first part of this study, the effect of long-term (7-day) treatment with clotrimazole (1, 3, 10, and 20 µmol/l) on Caco-2 and IEC-6 cellular viability, proliferation, and differentiation was investigated.

Clotrimazole (20 µmol/l) caused a significant decrease in both Caco-2 and IEC-6 cell viability, as shown by the increased levels of LDH in the extracellular medium (Fig. 1a).

The effect of clotrimazole on cell proliferation was determined by two distinct methods: the SRB assay and the ^{3}H-thymidine incorporation assay. According to the SRB method (which reflects cellular proliferation), clotrimazole caused a significant and concentration-dependent decrease in the cell growth, in both cell lines (although its inhibitory potency was slightly higher in IEC-6 cells; Fig. 1b). Together, the LDH and SRB results reveal that clotrimazole is cytotoxic to both tumoral (Caco-2) and nontumoral (IEC-6) intestinal epithelial cells, with a similar potency.

Fig. 1



Effect of a 7-day exposure to increasing concentrations of clotrimazole (1–20 µmol/l) on Caco-2 and IEC-6 cellular viability (a) and proliferation (b). Cells were seeded on 24-well plates. The cellular viability was determined by quantification of the extracellular lactate dehydrogenase activity, as described in Methods. The cellular proliferation was determined by quantification of the whole cellular protein with sulforhodamine B (SRB), as described in Methods. Results are shown as extracellular lactate dehydrogenase (LDH) activity (% of total LDH activity) (arithmetic mean + SEM; $n = 11–12$) (a) and as absorbance (% of control) (arithmetic means + SEM; $n = 8$) (b). *Significantly different from control ($P < 0.05$).

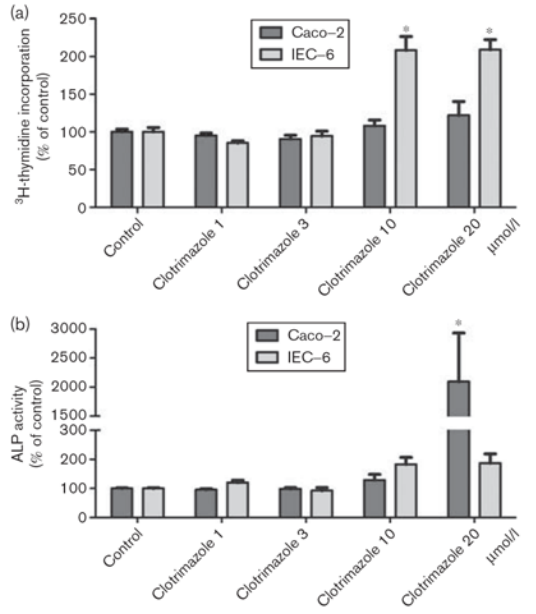
However, ^{3}H-thymidine incorporation [which occurs in the S (synthetic) phase, thus measuring the cellular DNA synthesis rate] was not affected by long-term (7-day) clotrimazole treatment in Caco-2 cells, but increased in the presence of clotrimazole (10 and 20 µmol/l), in IEC-6 cells (Fig. 2a).

Finally, the effect of clotrimazole on cellular differentiation was measured with the alkaline phosphatase activity assay. As can be observed in Fig. 2b, clotrimazole (20 µmol/l) caused a significant increase in Caco-2 cellular differentiation, but had no effect on the IEC-6 cellular differentiation status.

Effect of rhodamine123 and clotrimazole *on the cellular viability, proliferation, and differentiation of Caco-2 cells*

In the next series of experiments, we investigated the effect of clotrimazole, in conjunction with an inhibitor of mitochondrial oxidative phosphorylation (rhodamine123), on the viability, proliferation, and differentiation of

Fig. 2



Effect of a 7-day exposure to increasing concentrations of clotrimazole (1–20 μmol/l) on Caco-2 and IEC-6 DNA synthesis rate (a) and cell differentiation (b). Cells were seeded on 24-well plates. The DNA synthesis rate was determined by quantification of the incorporation of ^{3}H-thymidine, as described in Methods. The cell differentiation was determined by quantification of the alkaline phosphatase (ALP) activity, as described in Methods. Results are shown as μCi/well (% of control) (arithmetic mean + SEM; $n = 6$–9) (a) and as nmol *p*-nitrophenol/min/mg protein (% of control) (arithmetic mean + SEM; $n = 12$–16). (b). *Significantly different from control ($P < 0.05$).

Caco-2 cells. We decided to investigate this point because, in tumor cells, ATP production may be derived exclusively from glycolysis or from both glycolysis and oxidative phosphorylation, depending on the tumor type. Therefore, in some tumor cells, the sole application of glycolytic drugs does not decrease tumor progression significantly, but a combination of glycolytic inhibitors with antimitochondrial drugs such as rhodamine123 results in a strong increment in the anticarcinogenic effect (see Introduction). Rhodamine123 is a drug known to inhibit the growth of carcinoma cells but not normal cells *in vitro* and *in vivo* (e.g. [18,19,28]). It is a potent inhibitor of mitochondrial oxidative phosphorylation [29,30], through a strong inhibition of the key oxidative phosphorylation enzymes [28,30], thus inducing a reduction in the cellular ATP levels [19,31].

In preliminary experiments, the effect of a 7-day treatment with increasing concentrations of rhodamine123 (0.1, 1, 3, and 10 μmol/l) on cell proliferation was assessed with the SRB assay. Although rhodamine123 (0.1 and 1 μmol/l) were devoid of any effect, rhodamine123 (3 and 10 μmol/l) were found to decrease cell proliferation in a concentration-dependent manner (to 81 ± 7 and 49 ± %5 of control; $n = 10$–12). On the basis of this result, we selected rhodamine123 (3 μmol/l) for further experiments.

The effect of clotrimazole (3 μmol/l) alone, or in combination with rhodamine123 (3 μmol/l), on Caco-2 cell viability, proliferation, and differentiation was then examined. As shown in Fig. 3, clotrimazole and rhodamine123 caused no changes in the cellular viability and differentiation, but (20–40%) decreased cell proliferation significantly. Interestingly enough, the combination of clotrimazole with rhodamine123 was found to reduce cell viability significantly and to cause a more pronounced decrease in cellular proliferation, in relation to clotrimazole or rhodamine123 alone (Fig. 3).

Effect of clotrimazole *on the uptake of ^{14}C-BT, ^{3}H-DG, and ^{3}H-OMG by Caco-2 cells*

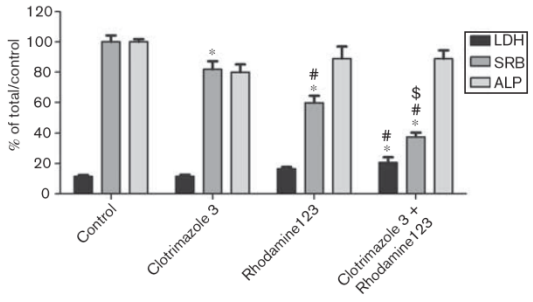
Finally, we decided to investigate the possibility that inhibition of glucose or butyrate uptake by Caco-2 cells contributes to the anticarcinogenic effect of clotrimazole in these cells. Our group previously showed that ^{14}C-BT and ^{3}H-OMG apical uptake in Caco-2 cells is linear with time for up to 3 min of incubation [25,32], and that ^{3}H-DG apical uptake is linear for up to 6 min of incubation [33]. Therefore, in the present study, cells were incubated with ^{14}C-BT and ^{3}H-OMG for 3 min and with ^{3}H-DG for 6 min to measure the initial rates of uptake.

The effect of clotrimazole on ^{14}C-BT, ^{3}H-DG, and ^{3}H-OMG apical uptake by Caco-2 cells was investigated in a first series of experiments. For this, 10 μmol/l of clotrimazole was tested over different time periods (1, 2, 3, and 7 days). Clotrimazole was devoid of a significant effect on the apical uptake of ^{14}C-BT (10 μmol/l; results not shown, $n = 12$). In contrast, a 1-day treatment with clotrimazole caused a 20% increase in the ^{3}H-DG uptake (Fig. 4a) and a 7-day treatment with clotrimazole caused a 30% increase in the ^{3}H-OMG uptake (Fig. 5a).

We further investigated the effect of a 1-day and a 7-day treatment with clotrimazole on the apical uptake of ^{3}H-DG and ^{3}H-OMG, respectively, by characterizing its concentration dependence. The effect of clotrimazole on ^{3}H-DG (Fig. 4b) and ^{3}H-OMG (Fig. 5b) uptake was concentration dependent, the maximal effect being observed with 10 μmol/l of the compound. This effect is not related to changes in the cell viability, because clotrimazole (10 μmol/l) caused no significant effect in this parameter in these cells.

Absorption of glucose from the intestinal lumen involves both a high-affinity, Na^+-dependent, and phloridzin-sensitive glucose cotransporter (SGLT1) and a Na^+-independent, low-affinity, and high-capacity facilitative GLUT2 [20,34]. Therefore, in an attempt to further characterize the effect of clotrimazole on ^{3}H-DG and

Fig. 3



Effect of a 7-day exposure to clotrimazole (3 μmol/l), rhodamine123 (3 μmol/l), or to a combination of both compounds (3 μmol/l of clotrimazole + 3 μmol/l of rhodamine123) on Caco-2 cellular viability [lactate dehydrogenase (LDH)], proliferation [sulforhodamine B (SRB)], and differentiation [alkaline phosphatase (ALP)]. The cellular viability was determined by quantification of the extracellular LDH activity, as described in Methods. Results are shown as extracellular LDH activity (% of total LDH activity; $n=15$). The cellular proliferation was determined by quantification of the whole cellular protein with SRB, as described in Methods. Results are shown as absorbance (% of control; $n=15$). The cell differentiation was determined by quantification of the ALP activity, as described in Methods. Results are shown as nmol *p*-nitrophenol/min/mg protein (% of control; $n=18$). Results are presented as arithmetic mean + SEM. *Significantly different from control; #Significantly different from clotrimazole (3 μmol/l); $Significantly different from rhodamine123 (3 μmol/l; $P<0.05$).

^{3}H-OMG uptake, we determined the effect of clotrimazole (10 μmol/l) in the presence of inhibitors of SGLT1 and GLUTs. In the control cells, ^{3}H-DG uptake was inhibited by phloridzin and by cytochalasin B (Fig. 4c), and ^{3}H-OMG uptake was inhibited by cytochalasin B only (Fig. 5c). These characteristics were maintained in the clotrimazole-treated cells.

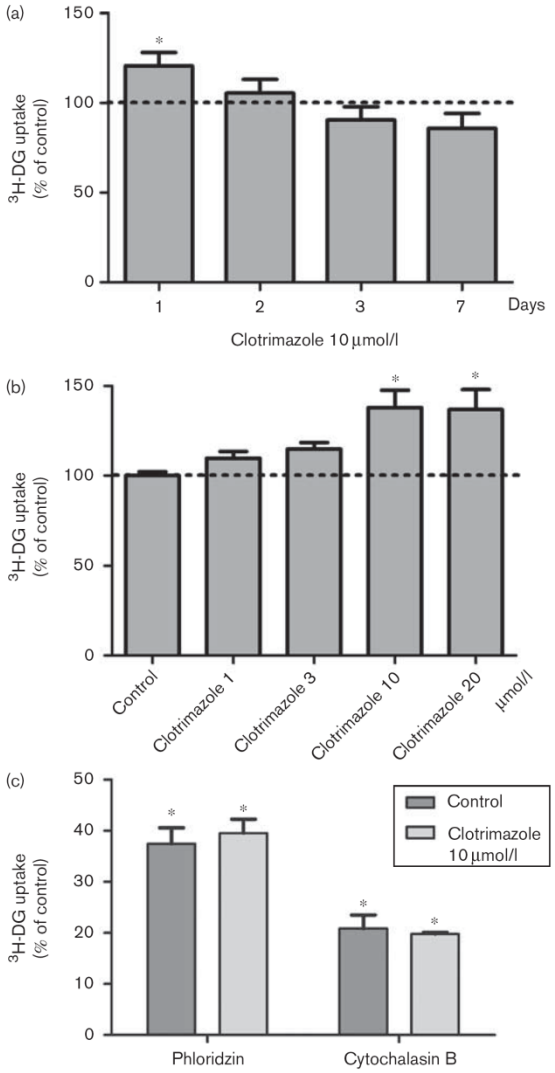
Qunatitative reverse transcription polymerase chain reaction

A comparison of the mRNA expression levels of SGLT1 and GLUT2 in the control and the clotrimazole-treated Caco-2 cells was carried out. Quantification of mRNA levels show that the expression of SGLT1 and GLUT2 mRNAs was significantly lower in the cells treated with clotrimazole for 1 day (Fig. 6). However, when cells were treated with clotrimazole for 7 days, the expression of SGLT1 mRNA did not change and there was a significant increase in the expression of GLUT2 mRNA, in relation to the control cells (Fig. 6).

Discussion

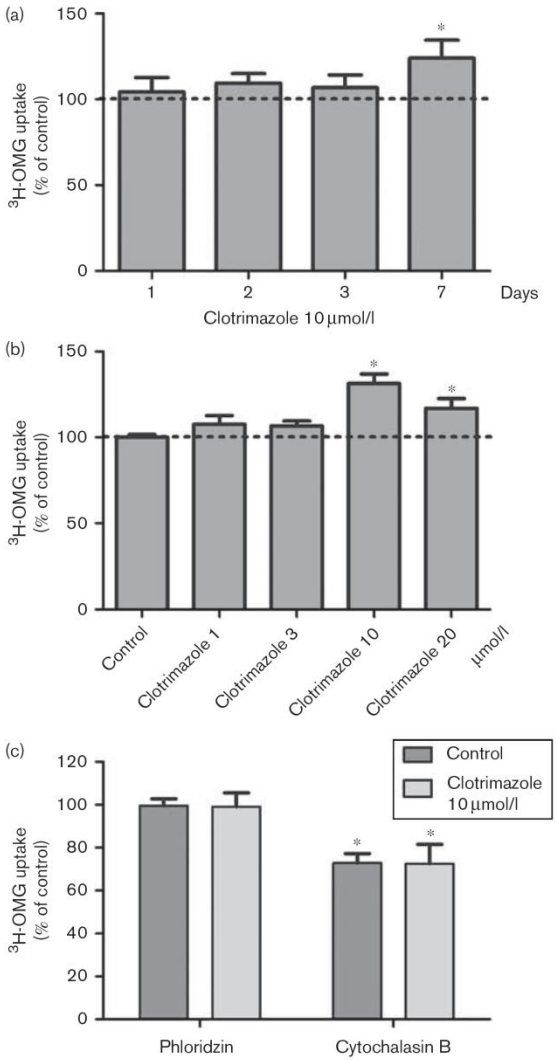
Clotrimazole is a therapeutically important imidazole-derived antifungal agent that is clinically safe and readily tolerated by humans. Its antifungal effect is due to the inhibition of ergosterol synthesis, which alters fungi plasma membrane permeability [35]. At the molecular level, clotrimazole has multiple effects on a variety of cellular targets, including an inhibitory effect on cytochrome

Fig. 4



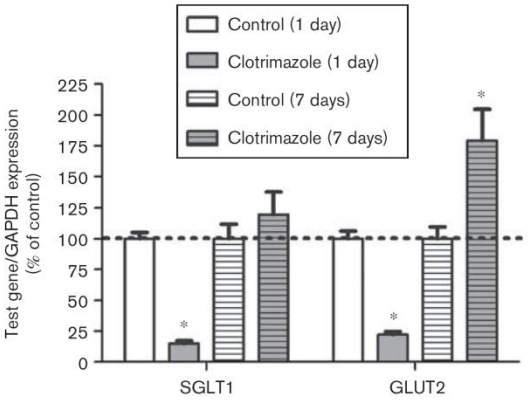
Effect of clotrimazole on the apical uptake of ^{3}H-deoxyglucose (^{3}H-DG) by Caco-2 cells. (a) Time dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) after treatment for 1, 2, 3, or 7 days with clotrimazole (10 μmol/l; $n=12$). (b) The concentration dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) after treatment for 1 day with increasing concentrations of clotrimazole (1–30 μmol/l; $n=12$–20). (c) Comparison of the effect of glucose transport inhibitors. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) in the absence or the presence of phloridzin (1 mmol/l) or cytochalasin B (100 μmol/l; $n=3$) after treatment for 1 day with clotrimazole (10 μmol/l) or its solvent (control). Results are presented as arithmetic mean + SEM (a and b) or as arithmetic mean ± SD (c). *Significantly different from the respective control ($P<0.05$).

Fig. 5



Effect of clotrimazole on the apical uptake of ^{3}H-O-methyl-glucose (^{3}H-OMG) by Caco-2 cells. (a) Time dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) after treatment for 1, 2, 3, or 7 days with clotrimazole 10 µmol/l ($n=12$). (b) The concentration dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) after treatment for 7 days with increasing concentrations of clotrimazole (1–30 µmol/l; $n=12$–20). (c) Comparison of the effect of glucose transport inhibitors. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) in the absence or the presence of phloridzin [1 mmol/l or cytochalasin B (100 µmol/l); $n=3$] after treatment for 7 days with clotrimazole (10 µmol/l) or its solvent (control). Results are presented as arithmetic mean + SEM (a and b) or as arithmetic mean ± SD (c). *Significantly different from the respective control ($P<0.05$).

Fig. 6



Quantification of mRNA levels of the human sodium-dependent glucose transporter (SGLT1) and of the human facilitative glucose transporter type 2 (GLUT2), by real-time quantitative reverse transcription polymerase chain reaction, after treatment of Caco-2 cells for 1 day or 7 days with clotrimazole (10 µmol/l) or its solvent (control; $n=5$). Results are shown as the expression of SGLT1 or GLUT2 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; % of control). Results are presented as arithmetic mean + SEM. *Significantly different from the respective control ($P<0.05$).

P450 [36], sarcoplasmic reticulum Ca^{2+}-ATPase [3,37], Ca^{2+}-dependent K^+ channels [38,39], and the Na^+, K^+-pump [40,41], and interference with cellular Ca^{2+} homeostasis [3]. Clotrimazole is also recognized as a calmodulin antagonist [42,43]. Calmodulin is a multifunctional Ca^{2+}-binding protein that has been implicated in the regulation of normal and abnormal cell proliferation [44,45], and calmodulin antagonists were reported to inhibit cellular proliferation in various cell types [42,46,47]. In agreement with this, clotrimazole was found to possess anticarcinogenic activity against different types of cancer. However, its anticarcinogenic effects at the intestinal level were reported previously only in a murine colon adenocarcinoma cell line (CT-26 cells) [5,6] and in a serum-independent HT29-S-B6 human colon adenocarcinoma cell clone [11]. Therefore, we found it important to investigate the anticarcinogenic effect of clotrimazole in a widely used human colon adenocarcinoma cell line, the Caco-2 cells [12], and to compare it with the effect in a nontumoral epithelial intestinal cell line (IEC-6 cells) [13].

In both Caco-2 and IEC-6 cells, clotrimazole (20 µmol/l) caused a decrease in the cell viability and proliferation (SRB assay). The effect of clotrimazole on the cell viability may be due to the detachment of glycolytic enzymes from the cytoskeleton [2] and/or inhibition of calmodulin activity [48]. The inhibitory effect of clotrimazole on cell proliferation is in agreement with previous studies, showing that clotrimazole inhibits the proliferation of both normal and cancer cell lines *in vitro*

and *in vivo* [2–10]. A number of mechanisms are known to be involved in the cell growth inhibitory action of clotrimazole, including activation of protein kinase R, induction of the phosphorylation of eIF2alpha, induction of the expression of wild-type p53, inhibition of protein synthesis at the level of translation initiation, and inhibition of cyclin-dependent kinase activity required for progression into the S phase, leading to a growth arrest in the G_0/G_1 cell cycle phases (e.g. [3,11,49,50]).

Short-term (16–24 h) clotrimazole treatment causes a decrease in cells entering the S phase [9,49,50]. However, analysis of ^{3}H-thymidine incorporation results suggests that, in Caco-2 cells, the decrease in cell proliferation induced by long-term (7-day) clotrimazole treatment does not result from a decrease in the cell DNA synthesis rate. Instead, it may result from an increase in cell death by necrosis or apoptosis (as clotrimazole induced human breast cancer cell apoptosis [7] and CT-26 murine colon adenocarcinoma cell necrosis [5,6]). The observation that high concentrations of clotrimazole decrease Caco-2 cell viability supports this hypothesis. In contrast, in IEC-6 cells, clotrimazole inhibited cell proliferation and increased the DNA synthesis rate. We hypothesize that apoptosis-induced compensatory proliferation, a process in which cell loss can induce additional divisions of the remaining cells, may be occurring in these cells (e.g. [51,52]).

In summary, clotrimazole showed cytotoxic activity in Caco-2 cells, by decreasing cellular viability and proliferation, and increasing cell differentiation. However, clotrimazole showed cytotoxic effects (reduction in cellular viability and proliferation) also in IEC-6 cells with a similar potency, although it increased the cellular DNA synthesis rate and had no effect on cell differentiation. Therefore, the anticarcinogenic potential of clotrimazole at the intestinal epithelial level is compromised by the fact that this compound also affects nontumoral cells.

Next, we evaluated the effect of clotrimazole in conjunction with an inhibitor of mitochondrial oxidative phosphorylation (rhodamine123), on the viability, proliferation, and differentiation of Caco-2 cells. In our experiments, rhodamine123 (3 μmol/l) caused no changes in cell death and differentiation, but induced a significant decrease in cell proliferation. Interestingly enough, the inhibitory effect of clotrimazole on viability and proliferation was significantly potentiated in the presence of rhodamine123. These results are consistent with previous works with rhodamine123 in combination with another inhibitor of glycolysis, 2-deoxy-D-glucose [17–19]. Taken together, these results suggest that glycolysis may not be the rate-limiting pathway for ATP production in colon cancer cells, and that administration of a drug that inhibits glycolysis (e.g. clotrimazole, tamoxifen, imatinib, cisplatin) in conjunction with an inhibitor of oxidative phosphorylation (e.g. 5-fluorouracil, taxol, diclofenac, sulindac) may be useful for treating colon tumors [16,53].

Finally, we decided to investigate the possibility that inhibition of glucose or butyrate uptake by Caco-2 cells contributes to the anticarcinogenic effect of clotrimazole in these cells. We verified that clotrimazole (10 μmol/l) did not affect ^{14}C-BT uptake, but caused a significant increase in ^{3}H-DG uptake at the first day and in ^{3}H-OMG uptake at the seventh day of treatment. Moreover, in both the control and the clotrimazole-treated cells, ^{3}H-DG uptake was phloridzin sensitive and cytochalasin B sensitive, and ^{3}H-OMG uptake was cytochalasin B sensitive but phloridzin insensitive. These final results suggest that, under the conditions of our study, the most important glucose transporter in Caco-2 cells seems to be a GLUT family member, and that clotrimazole does not alter the pharmacological characteristics of ^{3}H-DG and ^{3}H-OMG uptake significantly.

Next, we evaluated the effect of clotrimazole on the mRNA expression levels of SGLT1 and GLUT2. In contrast to the effect of clotrimazole on ^{3}H-DG uptake (increase), treatment of Caco-2 cells for 1 day with clotrimazole caused a marked decrease in the steady-state mRNA levels of both GLUT2 and SGLT1. This suggests that either (a) although clotrimazole decreases SGLT1 and GLUT2 mRNA levels, it increases GLUT2 and SGLT1 functional protein levels or activity, (b) clotrimazole affects other GLUT family glucose transporters, as human cancers of the digestive system have a marked increase in the mRNA levels of both GLUT1 and GLUT3 [54], and that well-differentiated Caco-2 cells express high levels of apical GLUT3 [55], or (c) the decrease in SGLT1 and GLUT2 mRNA levels are compensation mechanisms for the increase in ^{3}H-DG uptake induced by clotrimazole. The last hypothesis was advanced by Faria *et al.* [56], who found that an extract of anthocyanins inhibited glucose transport in Caco-2 cells while simultaneously increasing GLUT2 mRNA expression. In contrast, treatment of the cells for 7 days with clotrimazole markedly increased GLUT2 mRNA levels without changing SGLT1 mRNA expression. This increase in GLUT2 mRNA levels might well form the basis of the increase in ^{3}H-OMG uptake caused by clotrimazole. Therefore, these results suggest that the increase in ^{3}H-DG and ^{3}H-OMG uptake in the presence of clotrimazole constitutes a compensatory cellular mechanism in response to the inhibition of glycolysis caused by this agent. Moreover, they suggest that administration of a GLUT inhibitor (e.g. tamoxifen, cisplatin, etoposide) [16] might increase the anticarcinogenic effect of clotrimazole.

Conclusion

In Caco-2 cells, clotrimazole showed cytotoxic activity, decreasing cellular viability and proliferation, and increasing cell differentiation. The cytotoxic effect of clotrimazole was also observed in a nontumoral intestinal epithelial cell line (IEC-6 cells), although clotrimazole increased the cellular DNA synthesis rate and had no

effect on the differentiation of these cells. In Caco-2 cells, the effect of clotrimazole on cell proliferation and viability was markedly potentiated by rhodamine123, an inhibitor of mitochondrial oxidative phosphorylation. Finally, stimulation of glucose uptake might be a compensation mechanism in response to the glycolysis inhibition caused by clotrimazole in these cells.

Acknowledgements

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Conflicts of interest

None declared.

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DISCUSSION AND CONCLUSIONS

A - Characterization of BT transport at the intestinal epithelial level

BT is an important metabolic substrate in normal colonic epithelial cells; however, colonic epithelial tumoral cells show a reduction in BT uptake (through a reduction in MCT1 and SMCT1 protein expression) and glycolysis becomes their primary energy source [221,226]. So, knowledge on intestinal BT transport mechanisms and their regulation is crucial in the context of CRC pathophysiology. Moreover, BT presents an anticarcinogenic effect by induction of cell differentiation and apoptosis and by inhibition of proliferation, thus reducing the growth rate of many colon tumoral cells [56,57], and knowledge on this anticarcinogenic effect of BT led to a number of clinical trials testing its effectiveness as a potential treatment for cancer. Because the effects of BT (e.g. inhibition of histone deacetylases) are dependent on its intracellular concentration, knowledge on the mechanisms involved in its membrane transport is therefore relevant to both its physiological and pharmacological benefits. In this context, this study aimed at investigating the characteristics of the intestinal transport of BT, its regulation, and to search for implications of BT intestinal transport for CRC. Because *in vivo* studies performed with humans and laboratory animals are expensive, time-consuming and often even unethical (the three "Rs" paradigm), *in vitro* methods (as accurate as possible) offer a suitable alternative for *in vivo* testing. Primary colon cell cultures (isolated from human or animal tissue), are rarely used due to limitations with repeatability and long-term studies because of their short life span. So, in order to accomplish our objectives we studied the uptake of ^{14}C-BT in several immortalized intestinal epithelial cell lines. The great advantage of using these cell lines as a model is their relative easiness and inexpensiveness of culture and maintenance; long-term viability, reproducibility and uniformity; together with the suitability to allow performance of qualitative and quantitative transport studies, facilitating the study of many compounds and conditions. The cellular models used in this study were the Caco-2 human colonic adenocarcinoma cell line [254], and two nontransformed intestinal epithelial cell lines, the FHC fetal human colonic epithelial cell line [255] and the IEC-6 rat intestinal epithelial cell line [256]. We found important to compare BT transport in a colon adenocarcinoma cell line (Caco-2 cells) and in non-tumoral intestinal epithelial cell lines (FHC and IEC-6 cells) in the context of possible distinct BT transport mechanisms in these cells.

Previous studies have shown that MCT1 is expressed in normal colonic epithelium, that uptake of the primary energy source for these cells, BT, is MCT1-mediated [138-140], and that MCT1 expression decreases in colonic transition from normality to malignancy [66]. However, we have demonstrated that MCT1 plays a major role in the apical uptake of BT in the Caco-2 adenocarcinoma cell line, because ^{14}C-BT uptake by this cell line was: (1) time- and concentration-dependent; (2) pH-dependent; (3) Na^+-independent and Cl^--dependent; (4)

energy-dependent; (5) inhibited by several BT structural analogues (acetate, propionate, α-ketobutyrate, pyruvate, lactate); (6) insensitive to the anion exchange inhibitors DIDS and SITS and (7) inhibited by the MCT inhibitors NPPB and pCMB (**I**). This agrees with the fact that MCT1 is highly expressed in these cells (**I**) [138,257]. So, ^{14}C-BT uptake by Caco-2 cells is MCT1-mediated (Figure 13) and this cell line may be utilized to study regulation of MCT1 expression and function (**I**).

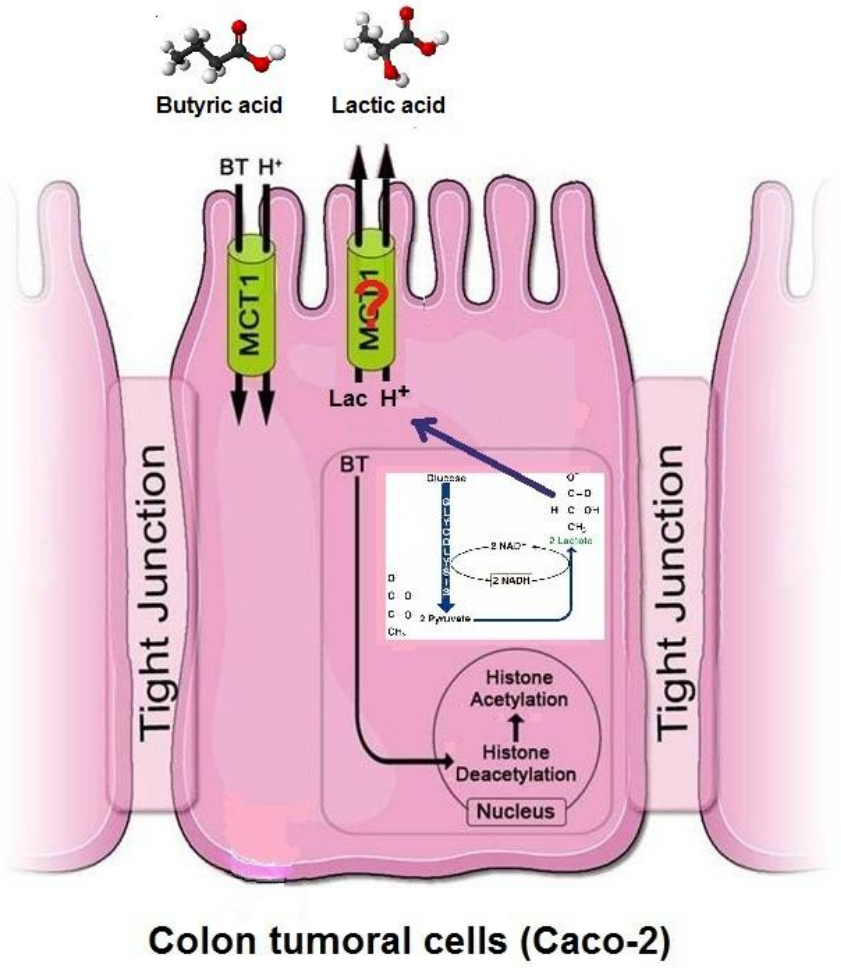

Figure 13. Proposed model of expression and function of BT transporters in the adenocarcinoma cell line Caco-2. In colon tumoral cells, glycolysis becomes the primary energy source, exceeding BT oxidative energetic metabolism [221,226], and cells rapidly convert the majority of glucose into lactate [258]. BT is a histone deacetylase inhibitor promoting histone acetylation. MCT1 (monocarboxylate transporter 1, gene name SLC16A1) mediates influx of BT and may also mediate efflux of lactate at the apical membrane.

Tumoral cells convert the majority of glucose into lactate, and to maintain a permissive intracellular pH, high glycolytic rates and ATP levels, cells must efficiently export lactate [244]. MCTs are bidirectional transporters [147], and powerful regulators of the tumor intracellular pH by extruding lactate together with a proton [244]. Although MCT1 expression decreases during colonic transition from normality to malignancy (being downregulated in the early

stages of carcinogenesis) [66], some studies also described an MCT1 upregulation in advanced metastatic CRC tumors [189,246]. In these advanced CRC tumors, cells are highly glycolytic and upregulation of some isoforms of MCTs may occur as a means of exporting lactate. Because the adenocarcinoma cell line Caco-2 expresses minimal levels of MCT4 [257], lactate may be extruded by MCT1 (Figure 13). Interestingly, MCT1 inhibition decreases intracellular pH, resulting in tumoral cell death [190,250,259]. The anticarcinogenic effect of BT is believed to result from inhibition of histone deacetylases [69,70]. However, it is also interesting to speculate that MCT1-mediated BT uptake may decrease the intracellular pH, resulting in tumoral cell death.

Studies have shown that SMCT1 expression is silenced in CRC and colon cancer cell lines, which may result in reduced uptake and metabolism of BT [67,201]. However, no studies have characterized SMCT1 function in the native human intestine, or in human nontumoral intestinal epithelial cell lines. So, we decided to characterize BT uptake by two nontransformed intestinal epithelial cell lines: the fetal human colonic epithelial (FHC) and the rat small intestinal epithelial (IEC-6) cell lines (**II**). The FHC cell line was found to express MCT1 but not SMCT1 mRNA. However, the characteristics of ^{14}C-BT uptake by FHC cells ((1) energy-independence; (2) pH-, Na^+- and Cl^-- independence and (3) insensitivity to BT structural analogues and MCT1 inhibitors) did not support either MCT1 or SMCT1 involvement in BT uptake. For this reason, FHC cells did not seem to be a good cell model to further investigate BT uptake (**II**). On the other hand, ^{14}C-BT transport characteristics in IEC-6 cells were as follow: (1) time- and concentration-dependence; (2) pH-dependence; (3) Na^+- and Cl^--dependence; (4) energy-dependence; (5) inhibition by BT structural analogues; (6) insensitivity to DIDS and amiloride; (7) sensitivity to MCT1 inhibitors (NPPB, pCMB and luteoline). Also, IEC-6 cells were found to express both MCT1 and SMCT1 mRNA. We thus conclude that ^{14}C-BT uptake by IEC-6 cells involves both MCT1 and SMCT1 (**II**). So, IEC-6 cells express a functional SMCT1 mediating BT absorption in a Na^+-dependent manner, and this cell line may be utilized to study regulation of SMCT1 expression and function (**II**). Interestingly, more recent kinetic experiments revealed the existence of a high affinity and a low affinity transporter for ^{14}C-BT in IEC-6 cells (**VII**), most probably corresponding to SMCT1 and MCT1, respectively (**II**) [139]. Physiologically, MCT1 is possibly more active in the proximal colon (K_m of MCT1 is about 2.4–2.8 mM of BT (**I**)), where there is a high concentration of BT (in the mM range after digestion of dietary fiber) and the pH is lower [65]. Because SMCT1 has a low K_m (50 µM), having a high affinity and low transport capacity for BT [260] and is more expressed in distal colon [193], SMCT1 is possibly more important in the distal colon (where there are lower concentrations of BT) [65]. However,

Smct1-null mice do not reveal a higher incidence of tumors in the colon [261], possibly because BT is also transported by MCT1 there [262].

BT cellular pools are not only dependent on the above BT uptake systems but also depend on efflux transporters, which are able to remove BT from the cells. The ATP-binding cassette (ABC) transporter superfamily includes membrane proteins that translocate a wide variety of substrates across membranes [263,264]. The human intestinal tract expresses high levels of some ABC transporters (e.g. P-glycoprotein (MDR1; encoded by ABCB1), multidrug resistance protein 1 (MRP1; encoded by ABCC1), and the breast cancer resistance protein (BCRP; encoded by ABCG2)), and these efflux transporters are believed to be involved in limiting drug absorption, bioavailability, and toxicity [263,264]. Because nothing was known concerning the putative interaction of BT with ABC transporter superfamily members, we decided to investigate the possibility of BT being transported by MDR1, MRPs or BCRP (**III**). Interestingly, we demonstrated that ^{14}C-BT is a BCRP substrate and that inhibition of BCRP significantly potentiates the effect of BT on cell proliferation (**III**). BCRP derived its name as a result of its isolation from the drug resistant breast cancer cell line MCF-7/AdrVp [265]. However, BCRP is also expressed abundantly in several normal human tissues, including the apical membrane of normal intestinal and colonic epithelium [266]. So, we propose that in normal colon epithelial cells, BT is influxed by MCT1 and SMCT1 at the apical membrane (**II**) [141,187] and, being the main energy source for colonocytes [53], is metabolized at the Krebs cycle. BT that is not metabolized is effluxed by BCRP, thus decreasing its intracellular concentration and so BT has no effect at HDACs levels (Figure 14). This is consistent with the fact that, in normal colonic tissue, BT does not inhibit cell proliferation [54,55,267-275].

BCRP expression is dramatically reduced in inflammatory bowel disease [276-278], which is interesting in the context of the relationship between inflammatory bowel disease and CRC, as cancer risk increases with the duration of inflammation [263]. Moreover, BCRP mRNA and protein expression is known to be significantly downregulated in human colorectal adenomas [263], in ApcMin mice (a mouse model of colon cancer) [263], in human CRC tissue and in most human cancers [279], suggesting that malignant transformation of the colonic epithelium *in vivo* is accompanied by a significant downregulation of BCRP. Studies also demonstrated that human BCRP polymorphisms (causing a decrease in its activity) could influence individual susceptibility to cancer [280,281]. Interestingly enough, BCRP exerts a protective function at the intestinal epithelial level by limiting the access of dietary mutagens and carcinogens, such as heterocyclic amines (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [282,283]; 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)

[284]), polycyclic aromatic hydrocarbons (benzo[a]pyrene [285]), micotoxins (aflatoxin B1 [286]), and sulfated and non-sulfated bile acids, to colonocytes [287]. So, a decrease in BCRP expression/function may place an individual at greater risk of exposure to dietary/environmental carcinogens.

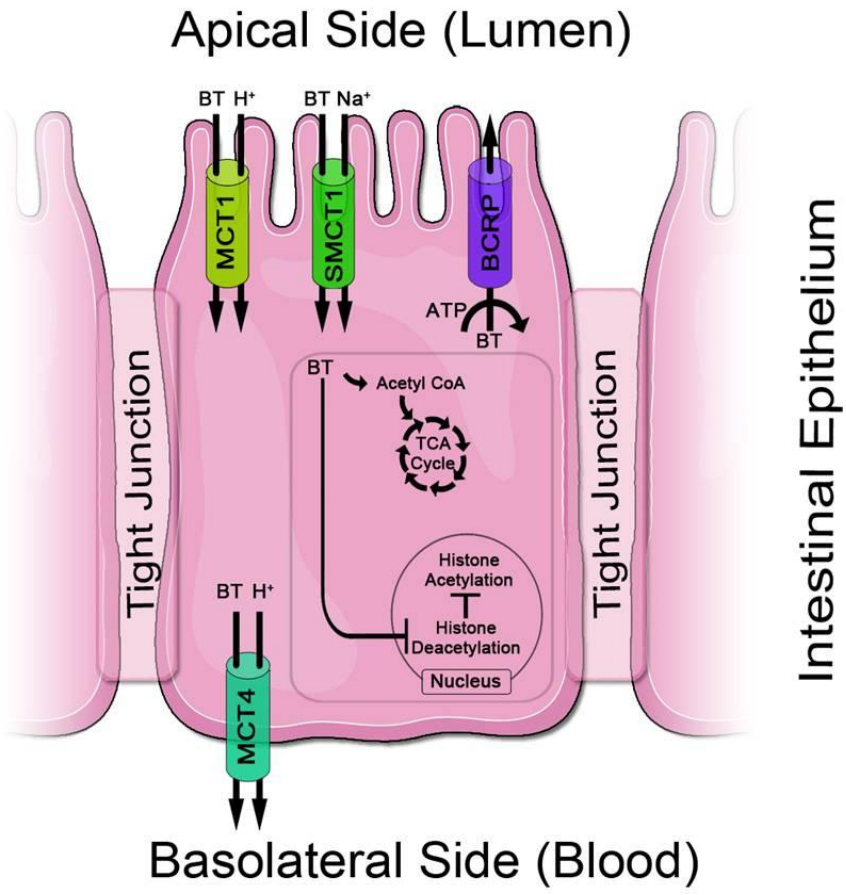

Figure 14. Proposed model of expression and function of BT transporters and its main intracellular targets in colonocytes, including its metabolism in the Krebs cycle and its effect on histone acetylation. Carriers such as MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane. BCRP (gene name ABCG2) is an ATP-dependent efflux transporter for BT at the apical membrane (**I**). At the basolateral membrane, efflux of BT occurs via MCT4 (monocarboxylate transporter 4, gene name SLC16A3).

As described above, CRC is associated with a decrease in BCRP expression [279]. In accordance with this, in our study, the tumoral Caco-2 cells did not show BCRP-mediated efflux of BT (**III**). So, we hypothesize that, in these cells, BT will act as a histone deacetylase inhibitor (HDAC), leading to hyperacetylation of histones and to increased accessibility of transcription factors to DNA promoters [70], thus inducing apoptosis (**V**) [56,92], inhibiting proliferation and promoting a more differentiated phenotype (**V**) [93,94]. These apparent contradicting effects

of BT in normal colonocytes and in tumoral cells are often referred to as the 'butyrate paradox' [94]. Differences in metabolism and transport of BT between normal and tumoral cells lines may contribute to this 'butyrate paradox' [288,289]. Based in our results, it is our opinion that the different levels of expression of BCRP in nontumoral and tumoral cells contributes to this paradox (**V**).

As already mentioned, BT inhibits cellular proliferation and induces cellular differentiation and apoptosis of tumoral cell lines [93,94]. However, some cells overcome this effect of BT and become chemoresistant to this agent, as evidenced by the existence of BT-resistant cell lines [270,290,291]. Given the anticarcinogenic effect of BT, identification of the mechanisms responsible for the acquisition of chemoresistance to this agent is of great potential value. As stated above, in our study, the tumoral cells (Caco-2) do not show BCRP-mediated efflux of BT (**III**), which is in agreement with the low expression of BCRP in these cells [292,293]. However, in BT-treated (48h) Caco-2 cells, BCRP expression appears to increase and resulted in BCRP-mediated efflux of BT (**III**). This effect of BT in inducing BCRP expression was previously verified with another BCRP substrate (imatinib) in this same cell line [294]. Moreover, some other histone deacetylase inhibitors (phenylbutyrate, valproate, trichostatin A, suberoylanilide hydroxamic acid, depsipeptide and romidepsin) also increase BCRP expression in cancer cell lines [295-297]. So, histone modifications are likely associated with drug-induced BCRP overexpression in cancer cells [296]. We thus suggest that the increase in BCRP expression levels in response to BT is involved in the acquisition of chemoresistance to BT in tumoral cell lines [270,290,291].

As stated above, CRC is associated with a downregulation of BCRP expression, at least in the early stages of carcinogenesis [279]. However, some studies described BCRP overexpression in colorectal invasive cancers (i.e., advanced stage of carcinogenesis) [298-300], suggesting that BCRP may be involved in cancer progression and metastasis. Numerous studies have also shown that BCRP is overexpressed in solid tumors [301]. Most solid tumors are exposed to low oxygen environments and BCRP gene transcription is activated by binding of HIF-1α to a hypoxia response element under low oxygen conditions [302]. Of note, BCRP is overexpressed in cancer cell lines and tumors that are "multidrug resistant" (MDR) (reviews by [303]). The term "multidrug resistance" (MDR) is used to describe the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs [304]. BCRP is one of the human ABC transporters that have been implicated in MDR in cancer chemotherapy [305,306]. BCRP recognizes and transports numerous anticancer drugs including conventional chemotherapeutic and relatively new molecules in clinical use: 5-fluorouracil, methotrexate, mitoxantrone, anthracyclines, daunorubicin,

doxorubicin, topotecan, diflomotecan, irinotecan, tyrosine kinase inhibitors (eg. imatinib and gefitinib) and nucleoside analogs [307-309]. Thus, downregulation of BCRP expression and/or function has been proposed as part of a regimen to improve cancer therapeutic efficacy. BCRP inhibitors can accelerate the endocytosis and degradation of BCRP in lysosomes [310,311]. Several specific inhibitors of BCRP have been reported, and some are currently undergoing clinical trials or are available to treat patients [301]. It is interesting to speculate that BT, by competing with anticancer agents for BCRP, can increase their intracellular level, thereby increasing their cytotoxicity. So, interaction of BT with BCRP and with other BCRP substrates/inhibitors is indeed of major importance [307-309], and the combination of BT and anticancer agents that are BCRP substrates might have clinical implications for CRC therapy [312].

Tumoral cells convert the majority of glucose into lactate, and to maintain a permissive intracellular pH, cells must efficiently export lactate [244]. So, as a consequence of a high rate of lactate production in tumors, the extracellular pH is more acidic (5.8) that in normal tissues (7.5) [313]. Curiously, BCRP is overexpressed in tumors exposed to acidic environment [298-300] and BCRP-mediated transport increases at acidic pH [314,315]. Because lactate is chemically related to BT and uses the same influx transporters [155,156], it would be interesting to evaluate if lactate is also effluxed by BCRP. Disruption of lactate transporters is one strategy for targeting tumoral cell metabolism [250-252], and if lactate is effluxed by BCRP, then inhibition of this transporter may be a promising target for anticancer strategy.

B - Modulation of BT transport and of its anticarcinogenic effect

Because BT plays a central role in colonic cellular metabolism and maintenance of tissue homeostasis, and because many cellular effects of BT are dependent on its intracellular concentration (e.g., inhibition of histone deacetylases), knowledge of the regulation of its absorption by the colon mucosa seems particularly important. Some studies have associated the intake of some mineral waters with reduced risk for CRC [316-318]. So, we decided to investigate the relationship between some mineral waters and ^{14}C-BT uptake by Caco-2 cells. Both acutely and chronically, the sodium-bicarbonate-fluorate-carbonic water (Vidago®) increased MCT1-mediated uptake of ^{14}C-BT (**IV**). Moreover, chronically, the bicarbonate-fluorate-carbonic water (Melgaço®) also increased MCT1-mediated uptake of ^{14}C-BT (**IV**). These results, showing that chronic exposure to Melgaço® and Vidago® water increases ^{14}C-BT uptake, suggests that chronic ingestion of these waters can have a protective role against CRC by increasing the intestinal epithelial uptake of BT (**IV**). Interestingly, Melgaço® water increases ^{14}C-BT uptake while simultaneously decreasing ^{3}H-O-methyl-glucose uptake (**IV**). The effect of

natural mineral waters upon ^{14}C-BT uptake is obviously dependent on their mineral content. Importantly, these mineral waters are rich in Ca^{2+} or Mg^{2+}, and some studies describe a protective effect of these minerals against CRC [319-321].

Coffee and tea consumption is associated with a lower incidence of CRC [322-324]. So, we decided to evaluate the effect of two xanthines present in these beverages, caffeine and theophylline, on ^{14}C-BT uptake (**I**, **II**). In Caco-2 cells, acute exposure to these xanthines reduced ^{14}C-BT (10 µM) uptake (**I**). So, we concluded that caffeine and theophylline are MCT1 inhibitors. However, chronic exposure to caffeine increased ^{14}C-BT uptake. The increase in ^{14}C-BT uptake is not related to changes in MCT1 gene expression level, but most probably results from an increase in MCT1 activity (**I**). Interestingly, caffeine increased ^{14}C-BT uptake while simultaneously decreasing Caco-2 cellular viability (**I**). However, in IEC-6 cells, caffeine was devoid of effect upon ^{14}C-BT uptake (**II**). Because BT uptake in Caco-2 and IEC-6 cells is MCT1-mediated and both MCT1 and SMCT1-mediated, respectively, these results suggest that acute caffeine inhibits MCT1 and has no effect upon SMCT1.

High intake of dietary fiber is associated with a reduced risk of CRC [45-48]. However, protective effects of fiber-containing food subgroups (e.g. fruits and vegetables) can also be contributed by the presence of other nutrients such as phytochemicals (plant secondary metabolites). Phytochemicals have reported anticarcinogenic effects in various *in vitro* and *in vivo* models, and have a potential chemopreventive role in CRC development [325]. BT (produced by fermentation of dietary fiber) and phytochemicals are both present in the colon and may directly or indirectly influence each other's actions. So, we decided to investigate the relationship between phytochemicals and ^{14}C-BT uptake by Caco-2 cells (MCT1-mediated) (**V**) and IEC-6 cells (MCT1 and SMCT1-mediated) (**II**). Phytochemicals consist of over four thousands of naturally occurring substances [325]. We focused our attention in studying the effects of some of the most abundant phytochemicals present in non-alcoholic (tea) and alcoholic (beer, red wine) beverages, as well as in several fruit and vegetables: catechin, epicatechin, epigallocatechin-3-gallate (EGCG), xanthohumol, resveratrol, quercetin, rutin, chrysin and myricetin.

Acutely (23 min), catechin, epicatechin and xanthohumol caused small but significant increases in ^{14}C-BT (10 µM) uptake by Caco-2 cells (MCT1-mediated) (**V**). On the contrary, some other phytochemicals (e.g. resveratrol, quercetin, myricetin and chrysin) inhibited ^{14}C-BT (10 µM) uptake (**V**). These phytochemicals were also tested against uptake of a high concentration of ^{14}C-BT (20 mM). We verified that resveratrol, quercetin, myricetin, chrysin, EGCG, and epicatechin, at least in some of the concentrations tested, were able to significantly reduce uptake of high concentration of ^{14}C-BT. For most of the compounds, the inhibitory

effect was not very pronounced, but resveratrol (100 µM) was able to cause a 30% reduction in ^{14}C-BT uptake. Resveratrol behaved as a competitive inhibitor of MCT1, since it reduced the transporter affinity for ^{14}C-BT (increased its K_m), without affecting its V_{max} (**V**). Resveratrol and quercetin also inhibited ^{14}C-BT uptake in IEC-6 cells (MCT1 and SMCT1-mediated), but myricetin and chrysin were devoid of effect (**II**). Because BT uptake in Caco-2 and IEC-6 cells is MCT1-mediated and both MCT1 and SMCT1-mediated, respectively, these results suggest that, acutely, resveratrol and quercetin do also inhibit SMCT1-mediated BT uptake and that myricetin and chrysin have no effect on this transporter.

However, when chronically tested (48h), some phytochemicals (e.g. quercetin, EGCG, rutin, chrysin, myricetin and catechin) were able to significantly increase ^{14}C-BT (20 mM) uptake by Caco-2 cells (**V**). Importantly, the increase in ^{14}C-BT uptake correlated with an increase in MCT1 mRNA expression levels for catechin (0.1 µM) and chrysin, suggesting that their effect upon ^{14}C-BT uptake results from changes in MCT1 transcription rates (**V**). As for quercetin, EGCG, rutin and myricetin, they probably change protein levels or the intrinsic transporter activity (**V**). This increase in ^{14}C-BT uptake might be involved in the anticarcinogenic effect of at least some of these phytochemicals (**V**). However, chrysin, myricetin and EGCG were devoid of effect when tested chronically, and resveratrol and quercetin even inhibited ^{14}C-BT uptake in IEC-6 cells, which was not associated with changes in MCT1 or SMCT1 transcription levels (**II**). So, the chronic effect of phytochemicals is dramatically different in Caco-2 and IEC-6 cells. Our general conclusion is that, chronically, phytochemicals increase ^{14}C-BT uptake (thus increasing its intracellular concentration) in tumoral cells (Caco-2), and have no effect or even inhibit ^{14}C-BT uptake in normal cells (IEC-6). These results are very interesting in the context of different effects of phytochemicals in tumoral and nontumoral cells.

In Caco-2 cells, BT (5 mM; 48h) markedly decreased cellular viability and proliferation and increased cell differentiation and apoptosis (**V**). Moreover, EGCG, quercetin, chrysin and rutin had no effect on cellular viability but significantly increased apoptosis (6–10x) (**V**). Also, EGCG, quercetin and chrysin showed antiproliferative effects at low concentrations (0.1-1 µM), but they presented a proproliferative effect when tested in higher concentration (10 µM) (**V**). The antiproliferative effect of EGCG, quercetin and chrysin may be associated with a decrease in ^{14}C-BT oxidation (by 13–15%) (**V**). Moreover, rutin decreased cell differentiation (**V**). We then investigated if some of these phytochemicals could interfere with the effects of BT upon cellular viability, proliferation, differentiation and apoptosis, and we verified that combination of these compounds with BT did not significantly modify the effects of BT (**V**). The lack of modulation of BT effects by phytochemicals might have two explanations: (1) chronic

treatment (48h) with BT (5 mM) upregulates MCT1 [158,168-170] and also MCT4 and its chaperone CD147 [326], and this increase in MCT-mediated transport maximizes BT uptake that is thus no further enhanced by phytochemicals; (2) chronically, phytochemicals such as quercetin and chrysin are known to increase the expression of efflux transporters such as BCRP [285,327]. So, besides increasing BT uptake by the cells, phytochemicals may also have caused an increase in its efflux (**III**), thus having no significant effect on its intracellular concentrations.

Nonsteroidal anti-inflammatory drugs (NSAIDs) emerged as a new perspective in tumor therapy as well as in cancer prevention [328,329]. Interestingly, uptake of some NSAIDs is in part mediated by MCT1 [330,331]. So, we decided to evaluate the effect of two NSAIDS, acetylsalicylic acid and indomethacin, on ^{14}C-BT uptake (**I**, **II**). In Caco-2 cells, acute exposure to acetylsalicylic acid and indomethacin concentration-dependently inhibited ^{14}C-BT uptake, and so we conclude that these two compounds are inhibitors of MCT1-mediated transport of BT (**I**). However, acetylsalicylic acid showed a very unusual effect, as it simultaneously increased the K_m and the V_{max} of ^{14}C-BT uptake, thus appearing to cause a decrease in the affinity while increasing the capacity of MCT1 for BT (**I**). In IEC-6 cells, indomethacin also inhibited ^{14}C-BT uptake (even more potently that in Caco-2 cells), but acetylsalicylic acid was devoid of effect (**II**). Because BT uptake in Caco-2 and IEC-6 cells is MCT1-mediated and both MCT1 and SMCT1-mediated, respectively, these results suggest that indomethacin also inhibits SMCT1-mediated uptake and that, on the contrary, acetylsalicylic acid has no effect upon this transporter.

Ethanol is the most frequently used drug worldwide [332]. Epidemiological data have identified chronic alcohol consumption as a significant risk factor for CRC [333,334]. Although ethanol is not carcinogenic in animal models, its bacterial fermentation in the colon produces acetaldehyde, which is highly toxic, mutagenic and carcinogenic, and the main responsible for alcohol-associated carcinogenesis [335,336]. Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH) present in intestinal bacteria and colonocytes, and then to acetate by aldehyde dehydrogenase (ALDH) in the liver. Of note, ADH activity is significantly higher in CRC tissues than in healthy tissues leading to the accumulation of acetaldehyde in CRC tissues after ingestion of ethanol [337]. Most of the acetaldehyde generated during alcohol metabolism *in vivo* is promptly eliminated by ALDH2 [338]. Interestingly, approximately 40% of Japanese, Koreans or Chinese carry a mutant allele in the ALDH2 gene (ALDH2*2) [338,339]. This allele codes for an enzyme with little activity, thus leading to high acetaldehyde concentrations after the consumption of even small amounts of alcohol [339]. When individuals with this allele chronically consume ethanol, a significant increased risk for CRC is noted [339-341]. Cigarette smoking has also been suggested to be one of the risk factors for CRC [13,342]. Nicotine, one of the major components of cigarette smoking, can stimulate cell proliferation and suppress

apoptosis of colon cancer cells [343-346]. Cocaine also enhances tumor growth [347,348]. On the other hand, amphetamine decreases tumors in animal models [349,350], and Δ-9-tetrahydrocannabinol (THC) shows antitumoral effects [351]. Because the above mentioned substances (ethanol, acetaldehyde, nicotine, Δ-9-tetrahydrocannabinol (THC), cocaine, amphetamine and ecstasy (3,4-methylenedioxymethamphetamine, MDMA)) may have a direct effect in CRC, we decided to investigate their effect upon ^{14}C-BT uptake. We verified that acutely, ethanol in IEC-6 cells (**II**) and acetaldehyde in Caco-2 and IEC-6 cells (**I, II**) inhibited ^{14}C-BT uptake in a concentration-dependent manner. From these results, we can speculate that interference with ^{14}C-BT uptake in the colonic epithelium might contribute to the CRC promoter effect of ethanol and acetaldehyde, associated with the consumption of alcoholic beverages. Of the others drugs of abuse tested, only THC and MDMA (48h) decreased ^{14}C-BT uptake and the expression level of MCT1 (by 20–30%) (**I**). So, we conclude that interference with BT uptake does not seem to be the main mechanism involved in the effect of these compounds upon tumor growth.

High dietary fat is associated with increased CRC risk; however, it should be noted that the type of fat is also important. A growing body of experimental, epidemiological and clinical evidence supports the contention that bioactive food components containing n−3 long chain polyunsaturated fatty acids (PUFAs) (e.g., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) decrease CRC incidence [352,353], and that the opposite effect is observed with n-6 PUFAs (e.g., linoleic acid (LA) and arachidonic acid (AA)) [354,355]. For this reason, we decided to investigate the effect of n-3 PUFAs (DHA and EPA), n-6 PUFAs (LA, γ-linolenic acid and AA), and conjugated linoleic acid (CLA) upon ^{14}C-BT uptake in tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines (**VI**). We observed only a discrete interference of n-3 and n-6 PUFAs and CLA with the apical uptake of ^{14}C-BT. So, we conclude that interference with the uptake of BT does not seem to be one of the mechanisms involved in the recognized role that these compounds have in colon carcinogenesis. The antitumoral activity associated with n−3 polyunsaturated fatty acids may depend more on (1) modulation of COX-2 activity; (2) alteration of membrane dynamics and cell surface receptor function, and (3) increased cellular oxidative stress levels [356].

A possible mechanism that associates dietary fat with increased CRC risk is the production of bile acids for solubilization and absorption of dietary lipids in the gut [357]. Several epidemiological, animal and *in vitro* studies correlate secondary bile acids with CRC [358]. In relation to primary bile acids, less information is available. However, the primary bile salt chenodeoxycholic acid (CDCA) was shown to be tumour promoting in some animal and cell culture studies [359,360] and a significantly higher faecal concentration of CDCA was seen in

patients with CRC/adenoma [361]. CDCA concentrations up to 700–800 µM have been reported in fecal waters [362,363], suggesting high concentrations in colon. So, we decided to investigate the effect of CDCA upon ^{14}C-BT uptake in tumoral (Caco-2) and non-tumoral (IEC-6) intestinal epithelial cell lines. We demonstrated that chronically, CDCA markedly and concentration-dependently inhibits ^{14}C-BT uptake by IEC-6 cells (IC_{50} = 120 µM) and, less potently, by Caco-2 cells (IC_{50} = 402 µM) (**VII**). Moreover, the results suggest that CDCA inhibits both MCT1 and SMCT1 (Figure 15), although the inhibitory effect of CDCA is more pronounced for SMCT1 than for MCT1 (**VII**).

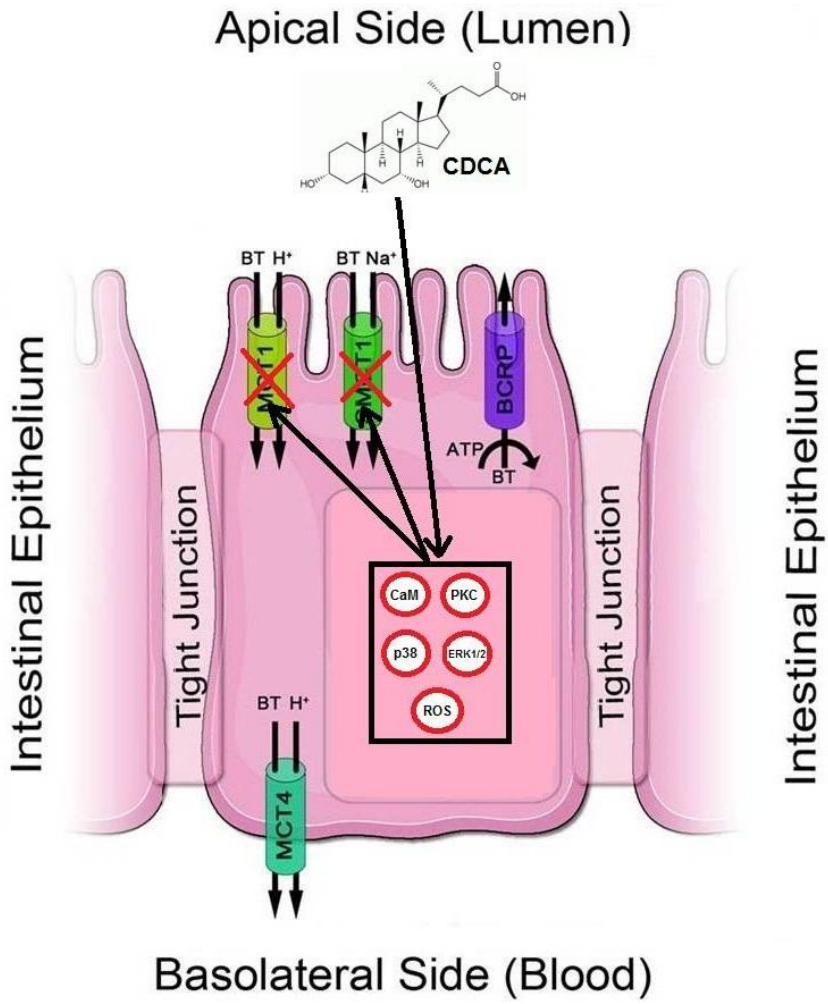

Figure 15. Effect of chenodeoxycholic acid (CDCA) in BT transport in intestinal epithelial cells. MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane. CDCA inhibits both MCT1 and SMCT1-mediated BT transport. Inhibition of BT uptake by CDCA is dependent on CaM, PKC, and MAP kinase (ERK1/2 and p38 pathways) activation and reduced by a reactive oxygen species scavenger.

Furthermore, CDCA reduces the effect of BT on cell viability and differentiation (**VII**), suggesting that inhibition of BT uptake by CDCA causes a lower intracellular concentration of this agent and less inhibition of HDACs. Moreover, inhibition of BT uptake by CDCA is

dependent on Ca^{2+}/calmodulin (CaM), mitogen-activated protein kinases (extracellular signal regulated kinase 1/2 (ERK 1/2) and p38 pathways) and protein kinase C (PKC) activation and is reduced by a reactive oxygen species (ROS) scavenger (**VII**). This last result suggests that bile acids-generated ROS [364,365] are involved in inhibition of ^{14}C-BT uptake by CDCA.

The gastrointestinal tract is a major target for oxidative stress damage due to constant exposure of ROS generated by a large variety of xenobiotics, the intestinal flora, and endogenous substances (e.g. bile acids) [366]. Also, production of ROS is a possible mechanism involved in alcohol-related carcinogenesis [367]. So, we decided to investigate the effect of oxidative stress upon BT uptake at the intestinal epithelial level. We chose to use IEC-6 cells because these are normal intestinal epithelial cells, and typically normal cells have persistently lower levels of ROS than tumor cells and are more sensitive to ROS [368]. IEC-6 cells were submitted to treatment with *tert*-butylhydroperoxide 3000 μM (tBOOH), which increased levels of oxidative stress biomarkers, while maintaining cellular viability. We demonstrated that oxidative stress inhibited SMCT1- mediated ^{14}C-BT transport, but not MCT1-mediated ^{14}C-BT uptake; moreover, it increases uptake and efflux of BT by passive diffusion (Figure 16) (**VIII**). Inhibition of SMCT1-mediated BT uptake by tBOOH is dependent on mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase 1/2 (ERK 1/2) and protein tyrosine kinase (PTK) activation and on the generation of ROS by NADPH and xanthine oxidase (**VIII**). Interestingly enough, the phytochemicals quercetin and resveratrol partially prevented the effect of oxidative stress upon ^{14}C-BT uptake (**VIII**). Quercetin and resveratrol are known to have strong antioxidant activities, due to their ability to directly scavenge ROS and to modulate expression of antioxidant enzymes [325]. Quercetin is also known to inhibit xanthine oxidase (which appears to be activated by tBOOH) [369]. Quercetin completely abolished the increase in lipid peroxidation caused by tBOOH (**VIII**). So, treatment with these phytochemicals possibly reduced cellular oxidative stress levels and thus protected the structure and function of BT transporters. Knowing that tumor cells are under increased ROS and ROS-induced oxidative stress compared to normal cells [370,371], and that oxidative stress inhibits ^{14}C-BT uptake (**VIII**) we can speculate that: (1) the absence of SMCT1-mediated ^{14}C-BT uptake observed in tumoral intestinal epithelial cells (Caco-2 cells) may be related to the increased oxidative stress levels found in these cells [370,371]; and (2) the increase in ^{14}C-BT uptake in Caco-2 cells found after chronic treatment (48h) with some phytochemicals (quercetin, EGCG, rutin, chrysin, myricetin and catechin) (**V**) is possibly related to their antioxidant properties (and consequent reduction in oxidative stress) [370,371].

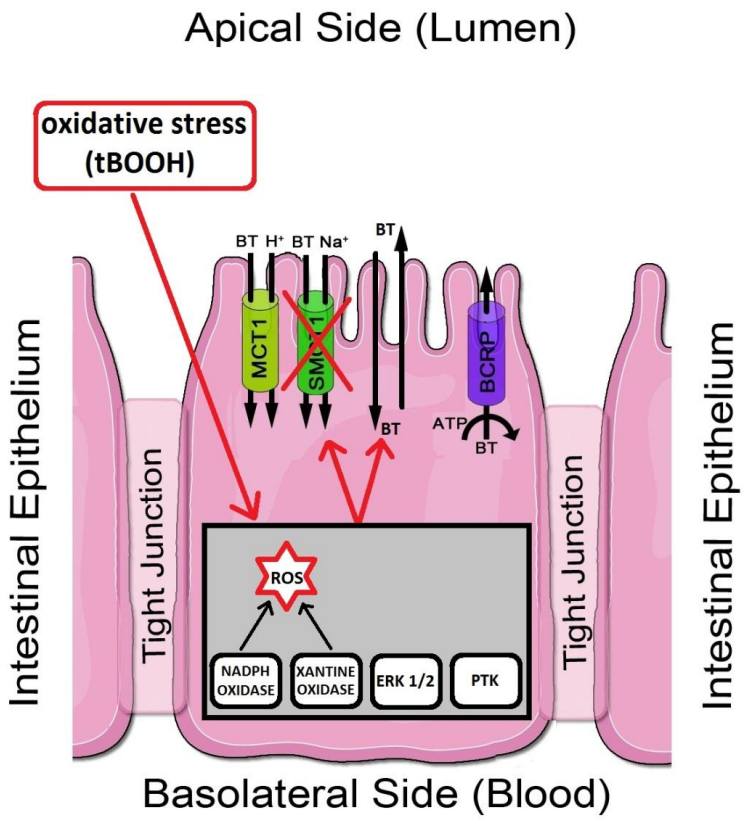

Figure 16. Effect of oxidative stress (tBOOH 3000 µM) on BT transport in intestinal epithelial cells. MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane. BCRP (gene name ABCG2) is an ATP dependent efflux transporter for BT at the apical membrane. Oxidative stress inhibits SMCT1-mediated BT uptake and stimulates both uptake and efflux of BT by passive diffusion. The inhibition of BT uptake is dependent on the generation of ROS by NADPH and xanthine oxidase, and on mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase 1/2 (ERK 1/2) and protein tyrosine kinase (PTK) activation.

Altogether, from the analysis of the above discussed results, two important experimental remarks arise. First, the observation that BT uptake and modulation in Caco-2 and IEC-6 cells are functionally different, these cell lines being good models to study MCT1- and SMCT1-mediated intestinal BT transport and function, respectively. Second, the observation that acute and chronic treatment of the cells with diverse xenobiotics did not produce parallel results and this should be taken into account when speculating about acute effects from chronic effects and *vice versa*. Curiously, some xenobiotics have opposite effects when tested acutely or chronically. For example, acutely, xanthines (caffeine and theophylline) (**I**), some phytochemicals (resveratrol, quercetin, myricetin and chrysin) (**V**) and NSAIDs (acetylsalicylic acid and indomethacin) (**I**) are inhibitors of MCT1-mediated BT uptake. However, chronic exposure (48h) to these compounds did not change (theophylline, resveratrol, acetylsalicylic acid and indomethacin) or even increased (caffeine, quercetin,

myricetin, chrysin, rutin, EGCG, catechin) ^{14}C-BT uptake. This increase in ^{14}C-BT uptake might be involved in the chronic anticarcinogenic effect of these compounds. However, the chronic increase in ^{14}C-BT uptake observed with these compounds is not related to changes in MCT1 expression because most of compounds have no effect or even decreased the MCT1 expression level (**I**, **V**).

As mentioned, one of the proposed beneficial effects of BT on human intestinal health is the prevention of colon carcinogenesis. Therefore, compounds that acutely decrease ^{14}C-BT uptake into intestinal epithelial cells are potentially detrimental to intestinal health and integrity. Nevertheless, in CRC tumors, glycolysis becomes the primary energy source and these compounds possibly also inhibit lactate release mediated by MCT1 with a consequent inhibition of glycolysis (**II**; **V**) [181-183]. So, we can speculate the inhibition of tumoral metabolism (i.e. glycolysis) is a mechanism accounting for their acute anticarcinogenic effects. Interestingly enough, some compounds described as acutely inhibiting MCTs have been pointed out as chemopreventive agents (e.g. BT, phytochemicals, NSAIDS, cholesterol synthesis inhibitors and lonidamine) [372-376]. Three very interesting cases of MCT1 inhibition and inhibition of glycolysis are exemplified below: 1) BT, which is transported by MCT1 (**I**), but is also known to decrease glucose uptake, glycolysis, glutaminolysis and lactate secretion [273,377,378]; 2) the phytochemical resveratrol, which is a competitive inhibitor of MCT1 (**V**), but is also described as a glucose uptake inhibitor [379], and was shown to decrease glycolysis in tumoral cells [380]; and 3) acetylsalicylic acid, which is an MCT1 inhibitor (**I**), and was shown to decrease glycolysis and lactate secretion in tumoral cells [381-383].

C - Cancer cell metabolism

The natural plant cannabinoid, Δ-9-tetrahydrocannabinol (THC), was recognized in 1975 as a potential anticancer agent [384]. Since then, there has been a great effort to investigate the therapeutic potential of cannabinoids in various types of cancer [385] and a first human dinical study has been performed in 2006 [351]. At the intestinal level, cannabinoid compounds show antitumoral effect by decreasing viability, proliferation, adhesion and migration in a variety of colorectal cancer cell lines (SW480, HCT-15, HT29, Caco-2, HCT116, LS174T and SW620 cells), as well as by modulating angiogenesis and metastasis [386,387].

A common feature of all cancers is their increased dependence on glucose as a preferred source for energy and biosynthesis [388]. So, colon tumoral cells show an increase in the rate of glucose uptake (glucose transporters, e.g. GLUT1) and glycolysis (the Warburg

effect) [221,224,225]. Accordingly, ample evidence indicates that cancer cells are more sensitive to glucose deprivation than normal cells [252,389,390]. Because THC has the ability to inhibit glucose uptake at testis, brain and placenta level [391-394], we decided to evaluate the effect of some cannabinoid agonists (THC, anandamide, CP55,940) and antagonists (AM251, AM630) on ^{3}H-2-deoxy-D-glucose (^{3}H-DG) uptake by the tumoral Caco-2 cells. 2-deoxy-D-glucose is a glucose analogue efficiently transported by facilitative glucose transporters (e.g. GLUT1) [395]. We verified that cannabinoid agonists had no effect in ^{3}H-DG uptake (**IX**). Acutely, the cannabinoid THC had also no effect in ^{14}C-BT uptake by Caco-2 cells (**I**), although chronic THC decreased ^{14}C-BT uptake and the expression level of MCT1 (by 30%) (**I**). So, we conclude that interference with the uptake of glucose and BT does not seem to be one of the mechanisms involved in the recognized role that cannabinoids have in colon carcinogenesis.

Clotrimazole (CTZ) shows anticarcinogenic activity in colon tumoral cells lines [396,397], associated with its ability to inhibit glycolysis and ATP production because it causes detachment of glycolytic enzymes from cytoskeleton [398]. We decided to investigate the anticarcinogenic effect of CTZ in a tumoral intestinal epithelial (Caco-2) cell line, to compare it with the effect in a nontumoral intestinal epithelial cell line (IEC-6 cells), and to investigate inhibition of energy substrate (glucose and BT) uptake as a mechanism contributing to it. In both Caco-2 and IEC-6 cells, CTZ (20 μM) caused a similar decrease in cell viability and proliferation (**X**). So, the anticarcinogenic potential of CTZ at the intestinal epithelial level may be compromised by the fact that this compound exhibits comparable cytotoxic effects in both tumoral and nontumoral intestinal epithelial cell lines. Exposure of Caco-2 cells to CTZ increased (by 20-30%) the uptake of glucose analogs (^{3}H-DG and ^{3}H-OMG) but had no effect on ^{14}C-BT uptake (**X**). We suggest that the increase in the uptake of glucose analogs in the presence of CTZ constitute a compensatory cellular mechanism in response to inhibition of glycolysis caused by this agent, and we also suggest that the inhibitory effect of CTZ in relation to glycolysis can be potentiated by a reduction in glucose availability through inhibition of its uptake (e.g. with phytochemicals that are able to effectively reduce glucose uptake and show anticancer properties [399-402]). Interestingly enough, combination of CTZ with rhodamine123 was found to significantly reduce Caco-2 cell viability and to cause a more pronounced decrease in cellular proliferation, in relation to CTZ or rhodamine123 alone (**X**). So, the cytotoxic effect of clotrimazole was strongly potentiated by inhibition of oxidative phosphorylation. Taken together, these results suggest that glycolysis may not be the rate limiting pathway for ATP production in Caco-2 cells, because oxidative phosphorylation is also active in these cells. Indeed, a growing number of studies have shown that mitochondria in

tumor cells are not inactive *per se* but operate at low capacity and produce ATP [403]. Also, in situations of glucose limitation, tumor cells can adapt to survive by using exclusively an oxidative energy substrate [404,405]. So, tumors show an enhanced glycolytic flux; however, not all have a diminished mitochondrial metabolic capacity. In conclusion, glycolysis and oxidative phosphorylation cooperate to sustain energy needs for Caco-2 cells [406]. Interestingly, glycolysis inhibition is particularly effective in those cells that present mitochondrial defects and rely most for their ATP production on this metabolic pathway [407], and targeting mitochondria might be a promising strategy to increase the sensitivity of tumor cells to apoptotic mechanisms [408]. So, administration of a drug that inhibits glycolysis (e.g. clotrimazole, tamoxifen, imatinib, cisplatin) in conjunction with an inhibitor of oxidative phosphorylation (e.g. 5-fluorouracil, taxol, diclofenac, sulindac) may be useful for treating colon tumors that have both active glycolysis and oxidative phosphorylation [408-410]. Other promising strategy is the use of compounds that simultaneously inhibit glycolysis and oxidative phosphorylation, e. g. 3-bromopyruvate [242] or resveratrol [379,411,412].

The final concluding remarks of this thesis are listed below.

With respect to the characterization of BT transport at the intestinal epithelial level:

(1) In the tumoral intestinal epithelial cell line (Caco-2 cells), ^{14}C-BT uptake is MCT1-mediated and this cell line may be used to study regulation of MCT1 expression and function.

(2) In the non-tumoral intestinal epithelial cell line (IEC-6 cells), ^{14}C-BT uptake is both MCT1 and SMCT1-mediated and this cell line may be used to study regulation of SMCT1 expression and function.

(3) BT is a breast cancer resistance protein (BCRP) substrate, and inhibition of BCRP significantly potentiates the effect of BT on cell proliferation.

With respect of the modulation of BT transport and of its anticarcinogenic effect:

(1) Acutely (23 min), MCT1 activity is inhibited by acetaldehyde, caffeine, theophylline, tetrahydrocannabinol and MDMA (ecstasy), by some phytochemicals (e.g. resveratrol, quercetin, myricetin and chrysin), and NSAIDs (acetylsalicylic acid and indomethacin), and is increased by some other phytochemicals (e.g. catechin, epicatechin and xanthohumol).

(2) Chronically (48h), MCT1 activity is increased by caffeine, quercetin, myricetin, chrysin, rutin, EGCG and catechin, and is inhibited by CDCA. SMCT1 activity is inhibited by CDCA and by oxidative stress.

(3) The expression of MCT1 mRNA is regulated by exposure to certain xenobiotics. Namely, it is decreased by EGCG, myricetin, and catechin, caffeine, tetrahydrocannabinol, MDMA (ecstasy), and increased by chrysin. SMCT1 mRNA expression is increased by CDCA.

(4) BT (5 mM; 48h) markedly decreased cellular viability and proliferation and increased cell differentiation and apoptosis of the tumoral intestinal epithelial cell line (Caco-2 cells).

(5) EGCG, quercetin, chrysin and rutin had no effect in cellular viability but significantly increased apoptosis (6–10x) of Caco-2 cells. EGCG, quercetin and chrysin showed antiproliferative effects at low concentrations (0.1-1 μM).

(6) Chronically (48h), EGCG, quercetin, chrysin and rutin increased ^{14}C-BT uptake in Caco-2 cells. However, combination of these compounds with BT did not significantly modify the effects of BT in cellular viability, proliferation, differentiation and apoptosis.

(7) CDCA inhibits both MCT1 and SMCT1-mediated ^{14}C-BT uptake and reduces the effect of BT on cell viability and differentiation of IEC-6 cells.

(8) Oxidative stress (tBOOH 3000 μM) inhibits SMCT1-mediated ^{14}C-BT uptake and stimulates both uptake and efflux of ^{14}C-BT by passive diffusion in IEC-6 cells.

With respect of cancer cell metabolism:

(1) Cannabinoid agonists have no effect in glucose uptake.

(2) In both Caco-2 and IEC-6 cells, clotrimazole (CTZ) caused a similar decrease in cell viability and proliferation. However, CTZ increased (by 20-30%) the uptake of glucose analogs (^{3}H-DG and ^{3}H-OMG) but had no effect on ^{14}C-BT uptake in Caco-2 cells.

(3) Glycolysis may not be the rate limiting pathway for ATP production in Caco-2 cells, because oxidative phosphorylation is also active in these cells.

With respect of the physiological implications of our results:

(1) SMCT1 is silenced in the tumoral intestinal epithelial Caco-2 cell line.

(2) Differences in BCRP expression between normal colonocytes and tumoral cells may contribute to different effects of BT in these cells ('the butyrate paradox').

(3) Interaction of BT with BCRP and with other BCRP substrates/inhibitors is of major importance, and the combination of BT and anticancer agents that are BCRP substrates might have clinical implications for CRC therapy.

(4) Administration of drugs that inhibit glycolysis (e.g. clotrimazole, tamoxifen, imatinib, cisplatin) in conjunction with an inhibitor of oxidative phosphorylation (e.g. 5-fluorouracil, taxol, diclofenac, sulindac) may be useful for treating colon tumors that have both active glycolysis and oxidative phosphorylation.

FUTURE PERSPECTIVES

We believe that the conclusions attained with this work contribute to a more profound knowledge on the mechanisms of BT intestinal transport and its modulation by diverse endobiotics and xenobiotics, its relationship to the anticarcinogenic effect of this compound and with CRC. As ever, in scientific research, much work remains to be done. In the near future, it would be interesting to evaluate *in vivo* (by using a rat model) the effect of some compounds/conditions that modulate BT uptake (e.g. ethanol, acetaldehyde, CDCA, oxidative stress) on the protective effects of BT against CRC [60,413]. Because we found BT to be a BCRP substrate, and BCRP acts as an efflux transporter for various anticancer agents (5-fluorouracil, methotrexate, mitoxantrone, anthracyclines, daunorubicin, doxorubicin, topotecan, diflomotecan, irinotecan, nucleoside analogs and tyrosine kinase inhibitors), and thus decrease their cytotoxic effects, the combination of BT and these anticancer agents might have clinical implications for CRC therapy. So, it would be very interesting to test this hypothesis in nude mice with tumor cells implanted in the colon [312].

Interestingly, tumoral colon cells convert the majority of glucose into lactate, and in order to avoid intracellular acidification and death, these cells must export protons [414]. Several systems are adapted for the transport of protons, among which MCTs should be considered candidates for pHi regulation and promising targets for anticancer strategies [251]. AZD3965, an orally administered compound related to AR-C117977 (a specific MCT1 inhibitor [415,416]) is currently entering Phase I/II clinical trials for advanced solid tumors therapy (http://science.cancerresearchuk.org/). However, systemic delivery of MCTs (and more specifically MCT1) inhibitors could affect almost every organ of the body, with the most drastic effects on cardiac and skeletal muscle (fatigue and toxicity) [417], and causing also inhibition of lymphocyte proliferation [184]. Also, silencing or pharmacological inhibition of MCT1 in colon cancer cells has been shown to be effective only in the absence of MCT4 expression, indicating functional redundancy of MCT1 and MCT4 [418]. However, no specific MCT4 small molecule inhibitor has been identified so far [415]. Because BT inhibits both transporters (MCT1 and MCT4) and is well metabolized, having no side effects [417], we think this molecule is a good compound to test in the context of inhibition of lactate release and glycolysis in CRC [419]. Interestingly, treatment of colon cancer cells with BT decreased glucose uptake, glycolysis, glutaminolysis and lactate secretion [273,377,378].

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SUMMARY

Butyrate (BT) is the main energy source for normal colonic epithelial cells and inhibits colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation). Studies suggest that BT is transported into colonic epithelial cells by the H^+-coupled monocarboxylate transporter 1 (MCT1) and by the Na^+-coupled monocarboxylate cotransporter 1 (SMCT1). However, colonic epithelial tumoral cells show a reduction in BT uptake, by lowering MCT1 and SMCT1 protein expression, and glycolysis becomes the primary energy source of these cells. So, knowledge on intestinal BT transport mechanisms and its regulation is crucial in the context of colorectal cancer (CRC) pathophysiology and of the physiological and pharmacological effects of BT. In this context, our objectives were to investigate the characteristics of intestinal transport of BT in the human colonic adenocarcinoma cell line Caco-2 and in two nontransformed intestinal epithelial cell lines, the fetal human colonic epithelial cell line FHC and the rat intestinal epithelial cell line IEC-6. We found important to compare BT transport in a colon adenocarcinoma cell line (Caco-2) and in non-tumoral intestinal epithelial cell lines (FHC, IEC-6) in the context of possible distinct BT transport mechanisms and regulation in these cells. Knowing that xenobiotics and endobiotics can modulate the risk for developing CRC, we aimed at studying regulation of BT transport mechanisms by these compounds. Finally, we also aimed at studying if some of the xenobiotics and endobiotics shown to interfere with BT transport would interfere with the anticarcinogenic effect of BT and if this effect can contribute to the anticarcinogenic or procarcinogenic effect of these compounds.

Our results led us to conclude that: 1) BT uptake by Caco-2 cells is MCT1-mediated; 2) BT uptake by FHC cells is neither MCT1 nor SMCT1-mediated; 3) BT uptake by IEC-6 cells is MCT1 and SMCT1-mediated; 4) BT is a substrate for the efflux transporter breast cancer resistance protein (BCRP), and inhibition of BCRP significantly potentiates the effect of BT on IEC-6 cell proliferation; 5) the tumor cells (Caco-2) do not show BCRP-mediated efflux of BT, but in BT-treated Caco-2 cells, BCRP-mediated efflux of BT was found. In relation to modulation of BT transport, we could conclude that BT transport is modulated by several chemically unrelated endo and xenobiotics, either after acute or chronic exposure. This may have important implications for CRC. For example, the decrease in BT uptake observed with ethanol, acetaldehyde, the primary bile salt chenodeoxycholic acid and with *tert*-butylhydroperoxide (oxidative stress inducer) and the increase in BT uptake found with caffeine and some phytochemicals (polyphenols) correlates well with their effects upon CRC. We also observed that the anticarcinogenic compound clotrimazole (CTZ), a glycolysis inhibitor, increases glucose uptake but does not affect BT uptake. So, the anticarcinogenic

effect of CTZ can be potentiated by a reduction in glucose availability through inhibition of its uptake.

We believe that these conclusions contribute to a more profound knowledge on the mechanisms of BT intestinal transport, its modulation by xenobiotics and endobiotics, and on the relationship between BT transport at the intestinal epithelial level and CRC.

RESUMO

O butirato (BT) é a fonte de energia principal para as células epiteliais do cólon normais e inibe a carcinogénese ao nível do cólon (pela supressão do crescimento das células tumorais, indução da diferenciação e apoptose e inibição da proliferação celular). Vários estudos sugerem que o BT é transportado para as células epiteliais do cólon pelo transportador de monocarboxilatos acoplado a H^+ do tipo 1 (MCT1) e pelo transportador de monocarboxilatos acoplado ao Na^+ do tipo 1 (SMCT1). No entanto, as células epiteliais colónicas tumorais apresentam uma redução na captação de BT (baixando a expressão proteica de MCT1 e SMCT1) e a glicólise torna-se a fonte de energia primária destas células. Assim, o conhecimento sobre os mecanismos de transporte intestinal de BT e a sua regulação é fundamental no contexto da fisiopatologia do cancro coloretal (CCR), e no contexto dos efeitos fisiológicos e farmacológicos do BT. Neste contexto, os nossos objetivos foram investigar as características de transporte intestinal de BT na linha celular de adenocarcinoma de cólon humano (células Caco-2) e em duas linhas celulares epiteliais intestinais não transformadas, a linha celular de epitélio fetal de colon humano (células FHC) e a linha celular de epitélio de intestino de rato (células IEC-6). A comparação entre o transporte de BT numa linha celular de adenocarcinoma de cólon humano (Caco-2) e em linhas celulares epiteliais intestinais não-tumorais (FHC, IEC-6) pareceu-nos importante no contexto de possíveis mecanismos distintos de transporte de BT e sua regulação nestas células. Sabendo-se que vários xenobióticos e endobióticos podem modular o risco para o desenvolvimento de CCR, tivemos também como objetivo estudar a possível interferência de alguns desses compostos com o transporte de BT. Finalmente, também foi nosso objetivo estudar como é que a interferência no transporte de BT pode contribuir para o efeito anti-carcinogénico ou pró-carcinógenos destes compostos.

Os nossos resultados levaram-nos a concluir que: 1) a captação de BT pelas células Caco-2 é mediada pelo MCT1; 2) a captação de BT pelas células de FHC não é mediada nem pelo MCT1 nem pelo SMCT1; 3) a captação de BT pelas células IEC-6 é mediada pelo MCT1 e pelo SMCT1; 4) o BT é substrato do transportador de efluxo proteína de resistência ao cancro da mama (BCRP), e a inibição da BCRP potencia significativamente o efeito do BT sobre a proliferação celular das células IEC-6; 5) as células tumorais (Caco-2) não mostram efluxo de BT mediado pela BCRP, mas em células Caco-2 tratadas com BT observa-se efluxo de BT mediado pela BCRP. Em relação à modulação do transporte de BT, podemos concluir que o transporte de BT é modulado por vários endo e xenobióticos não relacionados quimicamente, após uma exposição aguda ou crónica. Isto pode ter importantes implicações para o CCR. Por exemplo, a diminuição na captação de BT observada com o etanol, acetaldeído, o sal biliar primário ácido quenodesoxicólico e com o *tert*-butilhidroperóxido (um indutor de stresse oxidativo), e o

aumento na captação de BT encontrado com a cafeína e alguns fitoquímicos (polifenóis) correlaciona-se com o efeito destes compostos sobre o CCR. Também observamos que o composto anticarcinogénico clotrimazole (CTZ), inibidor da glicólise, aumenta a captação de glicose mas não afeta a captação de BT. Assim, o efeito anticarcinogénico do CTZ pode ser potenciado por uma redução na disponibilidade de glucose através da inibição da sua captação.

Acreditamos que estas conclusões contribuem para um conhecimento mais profundo sobre os mecanismos de transporte intestinal de BT, sua modulação por xenobióticos e endobióticos, bem como sobre a relação entre o transporte de BT a nível intestinal e o CCR.